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Nitric Oxide Synthase Activity in Pregnant Rabbit Uterus Decreases on the Last Day of Pregnancy

[Basic Science Section]

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Outline

Graphics

Abstract

OBJECTIVE: Our purpose was to test a potential role for the endogenous smooth muscle relaxant nitric oxide in the control of gestational uterine activity by quantifying and characterizing its synthetic enzyme, nitric oxide synthase, in uterine tissue at the end of pregnancy.

STUDY DESIGN: We measured nitric oxide synthase activity through the conversion of tritiated L-arginine to tritiated L-citrulline in subcellular preparations of decidua and myometrium from pregnant rabbits at 27, 30, and 31 days' (term)gestation. Nitric oxide synthase was characterized by measuring its relative inhibition by arginine analogs and its calcium-calmodulin requirement. Nitric oxide synthase activities were compared by one-way

analysis of variance with Fisher's post hoc test.

RESULTS: Nitric oxide synthase activity in decidua was high at 27 days' gestation (6.32 +-\1.10 pmol/mg protein per minute, n = 6), less with the approach of labor (30 days = 3.16 +-\1.25 pmol/mg per minute, n = 4), and lowest at 31 days (1.07 +-\0.29 pmol/mg per minute, n = 4, $p < 0.05$). Decidual nitric oxide synthase was calcium insensitive, and arginine analogs reduced activity with potencies consistent with their effect on the induced form of nitric oxide synthase.

CONCLUSION: Decidual nitric oxide synthase activity, which has the characteristics of the inducible isoform of the enzyme, is significantly lower on the last day of gestation. This suggests a role for nitric oxide in the control of uterine contractility during pregnancy. (AM J OBSTET GYNECOL 1993;169:1285-91.)

Key words: Rabbit, pregnancy, uterus, nitric oxide synthase, inducible isoform

Recent hypotheses and studies of the control of parturition have been directed primarily toward agents that stimulate uterine contractions. Increases in contractile stimulants, and myometrial sensitivity to these stimulants, are felt to be responsible for the extraordinary increase in uterine activity with labor [1]. But what keeps the phasically contracting uterine smooth muscle from delivering the fetus before the optimal term gestation? We asked whether there exists an endogenous uterine relaxant that fulfills this role.

One such endogenous relaxant, nitric oxide, is a potent second messenger in diverse organ systems [2]. In the circulatory system basal and stimulated production of endothelium-derived relaxing factor, nitric oxide, relaxes vascular smooth muscle, thus lowering vascular resistance [3]. Similarly, neurally produced nitric oxide is implicated in the relaxation of other smooth muscles, including the intestine and the corpora cavernosa of the penis [4]. Nitroglycerin and sodium nitroprusside, similar to nitric oxide, activate the soluble form of guanylate cyclase to relax smooth muscle [5]. These compounds have been used in obstetrics to reverse tetanic uterine contractions [6,7] or to relax the contracted postpartum uterus to facilitate placental extraction [8].

In addition to its role as a smooth muscle relaxant, nitric oxide subserves several other functions, including activity as a neurotransmitter [4] and immune mediator [2]. The latter role is especially relevant to a potential role in modifying the contractile response of the pregnant uterus. Murine macrophages contain a form of the enzyme nitric oxide synthase, which can be induced by endotoxin alone or in combination with cytokines to synthesize large quantities of nitric oxide for prolonged periods (>48 hours) [2,3]. Nitric oxide synthase induction can be prevented by protein synthesis inhibitors, glucocorticoids, or transforming growth factor-beta [2]. The decidua contains a high concentration of bone marrow--derived cells, which in the human are about 40% macrophages [9]. Vascular smooth muscle and

liver also contain an inducible form of nitric oxide synthase [2]. These inducible forms of the enzyme can be distinguished from the constitutive forms found in the nervous system and endothelial cells by their insensitivity to calcium-calmodulin concentration [2,3] and by their different relative sensitivity to inhibition by numerous N substituted L-arginine analogs [10]. Thus nitric oxide surrogates reduce uterine activity, and there is a potential source of inducible synthetic enzyme in decidua.

We hypothesized that decidual cells or myometrial smooth muscle cells contain nitric oxide synthase activity and that nitric oxide may be an endogenous uterine relaxant that decreases at the time of parturition. We report that nitric oxide synthase activity is present in decidua and decreases on the last day of gestation.

Material and methods

Tissue and cells. New Zealand White rabbits were time bred by the vendors (Grimaud Farms, Sunnyvale, Calif.) and killed at different gestational ages by pentobarbital overdose according to protocols approved by the University of California, San Francisco, animal care committee. In spite of timed breeding one rabbit expected to be at 30 days' gestation and one expected to be 31 days were delivered of pups before completing the thirtieth or thirty-first day. These were reassigned to a postpartum group. Hence the number of rabbits for each gestational age group was as follows: 27 days, n = 6; 30 days, n = 4; 31 days, n = 4; and post partum, n = 2. Cerebella from seven female nonpregnant rats were removed, immediately frozen, prepared as below, and stored at -70 degrees C. Human umbilical vein endothelial cells were harvested and cultured according to standard methods [11] and used at the third to sixth passages.

Subcellular fraction preparation. All tissues and cells were prepared according to the methods of Schmidt et al [12] and Bredt and Snyder, [13] except that the pellets and the supernatants were assayed. Briefly, tissue was homogenized with a TissueMizer (Tekmar) in buffer A containing Tris--hydrochloric acid, 50 mmol/L, pH 7.60, 0.5 mmol/L ethylenediaminetetraacetic acid, 0.5 mmol/L ethylene glycol bis(beta-aminoethyl ether), 1 micromole/L leupeptin, 1 micromole/L pepstatin A, 0.1 mmol/L phenylmethylsulfonyl fluoride, and 12.5 mmol/L 2-mercaptoethanol in a volume of 4 ml/gm of tissue (wet weight). Rabbit uterine homogenates were then centrifuged at 20,000g for 20 minutes (sufficient to sediment virtually all the nitric oxide synthase activity out of the supernatant), and rat cerebella homogenate was centrifuged at 100,000g for 1 hour [12]. The decidual pellets (containing nuclei, mitochondria, plasma membrane fragments, unbroken cells, and connective tissue) were washed by resuspension in four volumes of buffer and recentrifuged. The pellet was finally suspended in four volumes of buffer and homogenized with a Teflon pestle. To minimize loss of enzyme activity, all steps were carried out at ice-cold temperatures and assayed within 4 hours, except myometrium, which was frozen at -70 degrees C, and membranes were

prepared at a later date. Frozen homogenates lose one half their activity over 6 weeks (data not shown). To remove endogenous arginine, the particulates were washed once as noted above (able to remove 96% of tritiated L-arginine in tracer experiments), and experiments after dialysis were used to quantify residual endogenous arginine in representative preparations. Because of loss of enzyme activity with dialysis (see below), not all preparations were subjected to dialysis. Protein concentration was determined by the Bradford method with bovine serum albumin as the standard.

Assay of tritiated L-arginine conversion to tritiated L-citrulline. The stoichiometric production of nitric oxide was assayed by the conversion of (2,3,4,5- Hydrogen-3)-L-arginine to (2,3,4,5- Hydrogen-3)-L-citrulline after the methods of Bredt and Snyder [13] and Schmidt et al [12]. Duplicate tubes of 100 microliters containing 3 micromole/L unlabeled L-arginine, 100 to 200 nmol/L tritiated L-arginine (1,500,000 to 3,000,000 disintegrations/min), 1 mmol/L beta-nicotinamide adenine dinucleotide phosphate, reduced form, 2 mmol/L calcium chloride, 50 U/ml bovine calmodulin, 10 micromole/L (6R)H4-biopterin, and 5 to 10 mg/ml of protein were incubated for 30 minutes at 28 degrees C. Preliminary experiments indicated that enzyme activity under these conditions was linear with time (to 30 minutes) and enzyme protein concentration (to 12 mg/ml). The reaction incubation was stopped with 2 ml of iced buffer containing 40 mmol/L N-(22-hydroxyethyl)piperazine-N'-(22-ethane sulfonic acid), pH 5.50, and 4 mmol/L ethylenediaminetetraacetic acid. Tritiated L-citrulline produced was separated from unreacted tritiated L-arginine by passing the mixture over a 1 ml column of DOWEX AG50WX (prepared with 0.8 mol/L sodium hydroxide) and eluted with 2 ml of water. Gel phase scintillation spectroscopy of tritiated L-citrulline was performed by the external standard method to measure quenching and to allow calculation of absolute moles of citrulline produced. Control mixtures identical to the reaction mixtures, except without protein preparations, were similarly incubated and eluted through DOWEX AG 50WX. Resulting tritium counts (<4%), representing imperfect column retention of tritiated L-arginine and nonspecific conversion to other tritiated species, were subtracted from tritium counts per minute of every other reaction mixture elute.

Thin-layer chromatography of reaction products. We confirmed that the radioactive product of labeled L-arginine was lableled L-citrulline and not L-ornithine (from arginase conversion of arginine), by thin-layer chromatography on silica gel GF plates with methanol/chloroform/ammonium hydroxide 17% (2:2:1) solvent [14]. After a 60-minute incubation with 27-day decidual particulate (as above) 85.3% +-\1.8% (n = 3) of the resulting radioactivity ran with carrier L-citrulline, and with 31-day decidua 91.0% +-\0.6% ran with L-arginine (i.e., was not converted).

Assay of nitrite and nitrate production. Corroboration of the tritiated

L-arginine to tritiated L-citrulline assay of nitric oxide synthase activity was obtained by measurement of the stable oxidation products of nitric oxide, nitrite and nitrate, by an automated, copper-plated cadmium fillings high-pressure liquid chromatography column, followed by colorimetric reaction with the Griess reagent [15]. Similar reaction mixtures, but without radioisotope and with 100 micromole/L L-arginine, were incubated for up to 4 hours.

Characterization of nitric oxide synthase activity. Twenty-seven day and 31-day decidual homogenates were dialyzed with a Spectra/Por membrane (molecular weight cutoff 14,000 kd) against 100 volumes of buffer A for 2 hours at 4 degrees C. The 2 hours determined necessary to equilibrate arginine across the membrane resulted in a substantial loss of nitric oxide synthase activity: 16.0% +-\4.4% (n = 4) remaining versus 65.2% +-\7.9% (n = 4) remaining after merely maintaining the crude enzyme preparation in the same buffer at 4 degrees C for 2 hours.

Calcium-calmodulin sensitivity of nitric oxide synthase was assayed according to the method of Salter et al [16]: particulate enzyme preparations were incubated as above but with 2 micromole/L (Carbon-14)L-arginine (114,000 disintegrations/min) and 24 micromole/L L-arginine. Incubation including 2 mmol/L calcium per 50 U/ml calmodulin was compared with incubation with 1 mmol/L ethylene glycol bis(beta-aminoethyl ether) and without the addition of calcium-calmodulin.

The reaction was inhibited by serial incubations (as above) with increasing concentrations of N^G -methyl-L-arginine or N-omega-nitro-L-arginine methyl ester from 1 micromole/L to 10 mmol/L. The enzyme preparation was dialyzed as above to remove residual endogenous L-arginine. The inhibitor was added with cofactors precisely 10 minutes before L-arginine because inhibition is time and cofactor dependent [17].

Reagents. Tritiated L-arginine (62 Ci/mmol = 2.29 TBq/mmol) was obtained from Amersham; beta-nicotinamide adenine dinucleotide phosphate, reduced form, leupeptin, and pepstatin A from Boehringer-Mannheim; bovine calmodulin from CalBiochem; (Carbon-14)L-arginine (339 mCi/mmol = 12.6 GBq/mmol) and (Carbon-14)L-citrulline (55.9 mCi/mmol = 2.1 GBq/mmol) from New England Nuclear; (6R)H4-biopterin from Dr. B. Schricks (Jona, Switzerland); and all other chemicals from Sigma. Silica gel GF plates were obtained from Analtech (Newark, Del.).

Statistical analysis. Nitric oxide synthase activity is expressed as picomoles of total L-citrulline per milligram of protein per minute (mean +-\SEM) with n = number of different animals assayed. Means for each gestational age were compared by one-way analysis of variance with the Fisher post hoc test. For saturation analysis and enzyme inhibition assays Michaelis-Menten constants and concentration needed for 50% inhibitions were determined by a nonlinear iterative curve fitting program (Wavemetrics, Lake Oswego,

Ore.).

Results

Our preliminary experiments localized decidual nitric oxide synthase activity primarily to the particulate fraction. For example, in tissue from the same animal at 27 days' gestation the enzyme activity was 2.56 pmol/mg of protein per minute in the particulate fraction versus 0.17 pmol/mg of protein per minute in the cytosolic fraction. The difference was also present if the data were expressed as total activity per gram tissue (121.0 vs 13.8 pmol/gm of tissue per minute, respectively).

Nitric oxide synthase activity could also be detected in 27-day myometrium from the same animals, again primarily in the particulate fraction, but at considerably lower concentration (0.80 +-\0.17 pmol/mg of particulate protein per minute or 22.2 +-\4.7 pmol/gm of tissue per minute, n = 3) than in decidua from the same uteri (8.55 +-\0.71 pmol/mg of particulate protein per minute or 404 +-\47 pmol/gm of tissue per minute, n = 3).

Nitric oxide synthase activity at several gestational ages in decidual preparations is presented in Fig. 1. Nitric oxide synthase activity was highest at 27 days' gestation, at fivefold that of decidua obtained on the last day of pregnancy (31 days). Again the enzyme activity at 31 days' gestation was located primarily in the particulate fraction (data not shown). At 30 days of gestation results were more variable (3.16 +-\1.25 pmol/mg of protein per minute, minimum 1.16, maximum 6.80 pmol/mg of protein per minute, n = 4). Similar studies of myometrial particulate fractions on the last days of pregnancy indicated nitric oxide synthase activity at the lower level of detection of the assay, making it difficult to conclude how this activity changes at the end of gestation (data not shown).

The conversion of tritiated L-arginine to tritiated L-citrulline would be reduced if the specific activity of the isotope was reduced by endogenous arginine. Thus if endogenous arginine was substantially higher in preparations at 31 than at 27 days' gestation, it could result in an apparent difference in nitric oxide synthase activity with gestational age. To test this, we estimated the residual endogenous L-arginine concentration in 27- and 31-day rabbit decidua particulate. By comparing the apparent Michaelis-Menten constants before and after dialysis, one can estimate the concentration of residual endogenous arginine (Fig. 2). Before dialysis the Michaelis-Menten constant of decidua obtained at 27 days' gestation was 21.4 +-\2.4 micromole/L, compared with 2.10 +-\0.25 micromole/L in the same preparation after dialysis. From this difference we estimated the endogenous arginine in the 27-day decidual preparation as (approximately)20 micromole/L. In 31-day decidua the apparent Michaelis-Menten constants before and after dialysis (2.24 +-\0.65 micromole/L and 2.34 +-\0.61 micromole/L, respectively) indicate minimal residual endogenous arginine. Thus residual endogenous L-arginine, if having any confounding effect, would increase, not decrease, the difference

we found in nitric oxide synthase activity at 27 versus 31 days' gestation.

As a further test of decreased nitric oxide synthase activity at term, in one experiment we measured rabbit decidua generation of nitric oxide metabolites--nitrite and nitrate--from nonradiolabeled L-arginine. At 120, 180, and 240 minutes of incubation nitrite and nitrate generation was greater by particulate from 27-day than from 31-day animals (3.7 vs 1.0 micromole/L/mg of protein, 6.5 vs 1.5 micromole/L/mg of protein, and 6.1 vs 4.5 micromole/L/mg of protein, respectively).

Nitric oxide synthase activity was not calcium-calmodulin sensitive in decidual preparations obtained at 27 or 31 days' gestation. Without the addition of calcium-calmodulin the enzyme activities were 128% +-\12% (n = 4) and 144% +-\23% (n = 2), respectively, of the values obtained with the addition of calcium-calmodulin. In contrast, rat cerebellum nitric oxide synthase activity without calcium-calmodulin was 1.96% +-\0.92% of that with these cofactors.

The sensitivity of the enzyme to inhibition by arginine analogs was also quite different in decidua and cerebellum (Fig. 3). In cerebellum N^{G}-methyl-L-arginine was nearly equally effective as N-omega-nitro-L-arginine methyl ester (concentration needed for 50% inhibitions 13.3 +-\0.4 micromole/L and 10.8 +-\1.7 micromole/L, respectively). In decidual preparations from 27-day animals both analogs were considerably less effective, and N^{G}-methyl-L-arginine was tenfold more effective than N-omega-nitro-L-arginine methyl ester (concentration needed for 50% inhibitions 319 +-\89 micromole/L vs 2054 +-\740 micromole/L, respectively). Nitric oxide synthase activity was too low at other gestational ages and in other tissue sources to reliably compare inhibitors of activity.

Comment

Our results show that particulate preparations of the uterine decidua of pregnant rabbits have activity that conforms to the accepted behavior for nitric oxide synthase. Radiolabeled tritiated L-arginine is converted to a radiolabeled molecule with the cation exchange and thin-layer chromatography profile of citrulline. The conversion is inhibited by N-substituted arginine analogs, and the inhibition is reversed by an excess of L-arginine but not D-arginine (data not shown). Our nitric oxide synthase activity measurements of rat cerebellar cytosol (23.0 +-\2.2 pmol/mg of protein per minute) are comparable to published data of cerebellar cytosol nitric oxide synthase activity: 12 pmol/mg of protein per minute [12] and 160 pmol/mg of protein per minute [13]. An alternate pathway from arginine to citrulline is through the urea cycle. Urea cycle conversion of arginine to citrulline is very unlikely in our washed particulate decidual preparations because (1) substrate (glutamate) and cofactor (adenosine 5'-triphosphate) for the rate-limiting enzyme carbamoyl phosphate synthetase are depleted, (2) ornithine intermediate was not seen on thin-layer chromatography, and

(3) nitrite and nitrate (rather than only urea) were indeed produced as shown by the cadmium high-pressure liquid chromatography --Griess reagent assay.

Decidual tissue is very well vascularized, yet it is unlikely that the activity in decidua is accounted for solely by constitutive nitric oxide synthase in vascular endothelium. Decidual nitric oxide synthase activity is greater than that measured in pure cultures of human umbilical vein endothelium (Fig. 1), and more importantly the enzyme activity is characteristic of the inducible form of nitric oxide synthase as opposed to the constitutive form present in endothelium. Rabbit decidual nitric oxide synthase is insensitive to calcium-calmodulin, and N^G -methyl-L-arginine is a more potent inhibitor that is N-omega-nitro-L-arginine methyl ester. The inhibition of decidual nitric oxide synthase in the presence of calcium-calmodulin (the activity with no calcium-calmodulin >100% of the activity with calcium/calmodulin) is consistent with similar data reported for inducible macrophage nitric oxide synthase [18]. Although a membrane-bound nitric oxide synthase has been reported in macrophages, [19] it is calcium sensitive, unlike inducible nitric oxide synthase. All other reported inducible nitric oxide synthase isoforms are soluble [2]. Hence our finding of a rabbit decidual, membrane-bound, calcium-insensitive nitric oxide synthase may indicate a unique isoform of the enzyme.

The cellular localization of the nitric oxide synthase activity in the decidua is uncertain at present and awaits immunocytochemical characterization. The antibodies currently available against inducible nitric oxide synthase from murine macrophages [20] were all raised in rabbits and in our studies of rabbit tissue give unacceptably high background signals on Western blots of protein and on immunofluorescent histologic staining (data not shown).

The most striking aspect of these studies is the reduction of nitric oxide synthase activity, approximately 80%, that occurs on the last day of gestation in rabbits. Because our reaction incubation contained 3.1 micromole/L total added L-arginine and we measured the Michaelis-Menten constant of rabbit decidual nitric oxide synthase after dialysis to be 2.10 +-\0.25 micromole/L, the difference between 27- and 31-day nitric oxide synthase activity could be accounted for by increased maximum velocity or by increased enzyme affinity at 27 days' gestation. The data presented in Fig. 2 make the latter explanation unlikely, because under the conditions in which these assays were performed (no dialysis) the apparent nitric oxide synthase affinity of the 27-day sample was actually less (Michaelis-Menten constant was higher).

Results of nitric oxide synthase activity at day 30 were not merely variable but were either high as at day 27 or low as at day 31, suggesting a rapid switch in activity. Interestingly, two rabbits at 30 days' gestation instinctually preparing for delivery by pulling out their fur for a nest had low nitric oxide synthase activity as at 31 days' gestation, whereas the other 30-day gestation rabbits, not nesting, had higher nitric oxide synthase activity similar to results at 27 days' gestation. The signal for the reduction

of nitric oxide synthase activity is the subject of continuing studies. However, the apparent rapidity of the change is comparable to the striking changes in oxytocin receptors in rabbit myometrium at the end of gestation, which we and others have previously shown can be accounted for by progesterone withdrawal [21]. Little is currently known about the physiologic down-regulation of nitric oxide synthase other than the ability of glucocorticoids and transforming growth factor-beta to prevent cytokine-mediated induction [2]. Additional interesting candidates for the control of the decidual enzyme are prostaglandins, [22] cytokines such as interleukin-1, [23,24] and tumor necrosis factor [2,25].

We were able to demonstrate nitric oxide synthase activity in rabbit myometrium, although much less than in decidua, and this activity was not reliably different at the different gestational ages tested. The intimate association of decidua and the subjacent myometrium, and the high levels of activity in the decidua, are compatible with a role for decidual nitric oxide to influence myometrial contractions (by diffusion) in spite of the lability of the compound.

Because of the temporal relationship of the change in nitric oxide synthase activity to the onset of parturition, it is tempting to propose a necessary or sufficient role for the regulation of this activity in the maintenance of pregnancy and the onset of parturition. However, in previous studies by others testing the role of nitric oxide in the modification of hemodynamic responses during pregnancy, intravenous doses of nitric oxide synthase antagonists have not induced preterm parturition in rats [26,27,28]. The role of the striking alteration of decidual nitric oxide synthase activity in pregnancy and parturition, and its interaction with the many other mechanisms altering uterine contractility are yet to be determined.

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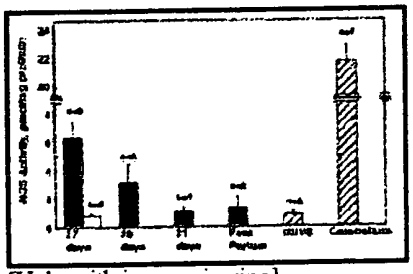

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Figure 1. Nitric oxide synthase (NOS) activity in pregnant rabbit uterus. Black bars, Decidual particulate fraction; white bar, myometrial particulate fraction; hatched bars, human umbilical vein endothelial (HUVE) cell particulate fraction and rat cerebellar cytosol as indicated. Data are means with number of animals indicated, and error bars are equal to SEM. Each is significantly different from 27-day decidua by one-factor analysis of variance and Fisher post hoc test ($p < 0.05$)

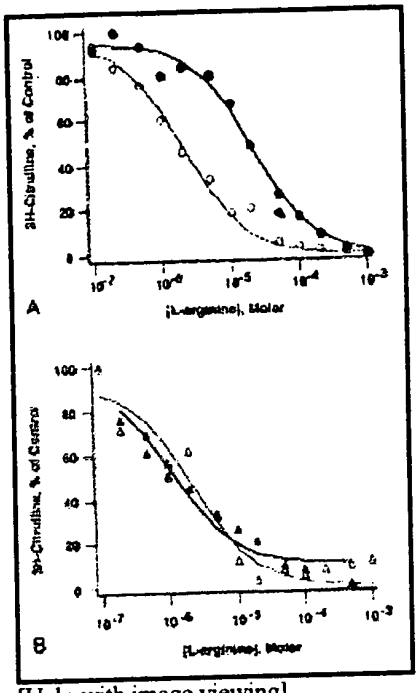

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Figure 2. Effects of dialysis on inhibition of nitric oxide synthase formation of radioactive citrulline by nonradioactive arginine. A, Open circles, 27-day decidua dialyzed; closed circles, undialyzed. B, open triangles, 31-day decidua dialyzed; closed triangles, undialyzed. Data are presented as tritiated L-citrulline production as percentage of control (tritiated L-citrulline produced with no added L-arginine) at indicated concentration of nonradioactive L-arginine. Symbols are actual data, and lines are curves of best fit. For decidua at 27 days, controls were 92.6 fmol/mg of protein per minute undialyzed (solid line) and 39.9 fmol/mg of protein per minute dialyzed (dotted line). Similar data for 31-day decidua were 4.23 fmol/mg of protein per minute undialyzed (solid line) and 3.23 fmol/mg of protein per minute dialyzed (dotted line)

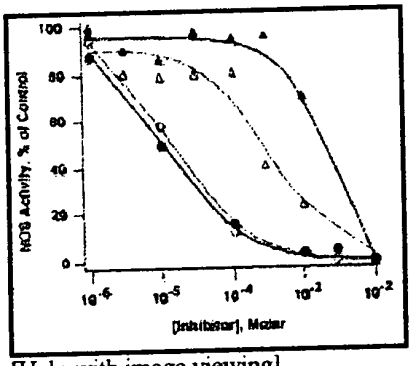

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Figure 3. Inhibition of nitric oxide synthase (NOS) by substituted arginine analogs. Serial incubations contained increasing concentrations (1 micromole/L to 10 mmol/L) of N^G -methyl-L-arginine (dashed line) or N-omega-nitro-L-arginine methyl ester (solid line). Enzyme preparation was dialyzed as in Fig. 2 to remove any extra endogenous arginine. Symbols are actual data, and lines are curves of best fit. Solid triangles, 27-day decidua plus N-omega-nitro-L-arginine methyl ester; open triangles, 27-day decidua plus N^G -methyl-L-arginine; solid circles, cerebellum plus N-omega-nitro-L-arginine methyl ester; open circles, cerebellum plus N^G -methyl-L-arginine

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The Acute Effect of Cocaine Exposure on Pregnant Human Myometrial Contractile Activity

[Transactions Of The Thirteenth Annual Meeting Of The Society Of Perinatal Obstetricians]

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Outline

Graphics

Abstract

OBJECTIVE: The objective of this study was to test the hypothesis that cocaine acutely increases contractile activity in isolated pregnant human myometrium.

STUDY DESIGN: Myometrial samples were obtained from the lower uterine segment at elective cesarean section from five women at term who were not in labor and who had no perinatal risk factors. Myometrial strips were suspended in contractile buffer, and isometric contractions were measured. Frequency, amplitude, duration, and integrated area (mean +-\SE) were compared before and after the addition of cocaine (10 sup -6 to 10 sup -4 mol/L) by means of analysis of variance and Duncan's multiple range test.

RESULTS: Contraction duration, expressed relative to control, increased acutely after

addition of cocaine (10 sup -5 mol/L, 2.0 +-\0.29; 10 sup -4 mol/L, 2.8 +-\0.64) ($p < 0.001$). Integrated area of contractions also increased relative to control (10 sup -6 mol/L, 1.6 +-\0.18, $p < 0.05$; 10 sup -5 mol/L, 2.4 +-\0.16 and 10 sup -4 mol/L, 3.5 +-\0.23, $p < 0.001$). These effects were dose dependent.

CONCLUSION: Cocaine acutely increases contractile activity in myometrium isolated from pregnant women. (AM J OBSTET GYNECOL 1993;169:782-5.)

Key words: Cocaine, myometrium, human, contractions, preterm labor

The last decade has seen a dramatic increase in the use of cocaine in pregnant women. It has been reported that 17% of women admitted to an inner-city hospital in labor gave a positive history of cocaine use at least once during pregnancy [1]. In a similar population 11% of parturients had a positive urine screen for cocaine metabolites, confirming recent use [2]. This widespread use has been associated with an increased risk of adverse perinatal outcome, including abruptio placentae, congenital anomalies, intrauterine growth restriction, and preterm labor [3]. The incidence of preterm delivery in cocaine users ranges from 20% to 50% in these women [3,4].

In spite of this well-recognized association of cocaine with preterm labor and delivery, little is known about the effect of cocaine on myometrium. Daniel [5] demonstrated that cocaine produced tetanic contractions in myometrium isolated from the pregnant cat and the nonpregnant rabbit but observed no effect on myometrium isolated from the nonpregnant rat. Hurd et al [6] reported that cocaine alone did not augment pregnant rabbit myometrial contractions but potentiated the response to epinephrine. It appears that there may be varied myometrial contractile response to cocaine between species. This study was performed to determine the effect of cocaine on the spontaneous contractile activity of isolated pregnant human myometrium.

Material and methods

Consent was obtained from women who were scheduled to have an indicated cesarean section at term in accordance with the Committee for the Protection of Human Subjects at the University of Texas at Houston. Only women who were not in labor and who had uncomplicated pregnancies were approached. After delivery of the fetus and the placenta a small myometrial sample was surgically removed from the upper edge of the lower-segment transverse uterine incision. The endometrial surface was gently dissected away, and longitudinal myometrial strips (four per sample) suspended in buffer [7] (composition in mmol/L: sodium chloride 125, potassium chloride 2.4, calcium chloride 1.8, magnesium chloride 0.5, sodium bicarbonate 23.9, and glucose 11.0) in jacketed tissue baths aerated with 95% oxygen and 5% carbon dioxide at 37 degrees C (pH 7.4). The strips were allowed to equilibrate at 1 gm tension for 20 to 30 minutes before the addition of

cocaine hydrochloride (Sigma, St. Louis) to the bath. Isometric contractions were monitored by force-displacement transducers (Grass FTO3, Grass, Quincy, Mass.) connected to a Beckman R411 dynograph recorder (Beckman, Palo Alto, Calif.). The recorder was calibrated so that 1 gm tension was represented as 1 cm vertical displacement. Paper speed was set at 0.1 mm/sec.

Contractions were measured over 1000-second intervals immediately before and after the addition of cocaine (10 sup -6 to 10 sup -4 mol/L). The frequency of contractions per 1000 seconds, the mean duration (seconds), and the amplitude (grams) of each contraction were measured. The integrated area under the contraction curve was calculated with a digitized planimeter. Data were expressed relative to control, reported as mean +-\SE, and analyzed with analysis of variance of log-transformed data and Duncan's multiple range test.

Results

Myometrial samples from five women were obtained. Median gestational age was 39.5 weeks. The indication for cesarean section was previous cesarean section (n = 4) and partial posterior placenta previa (n = 1).

Cocaine exposure acutely increased myometrial contractile activity Figure 1. Duration increased over control in a dose-dependent manner, reaching statistical significance at cocaine concentrations of 10 sup -5 and 10 sup -4 mol/L. Integrated area increased significantly after the addition of each concentration of cocaine Figure 2. There was no statistically significant effect on frequency or amplitude of contractions. Although there was a trend toward increase in amplitude, it did not reach statistical significance.

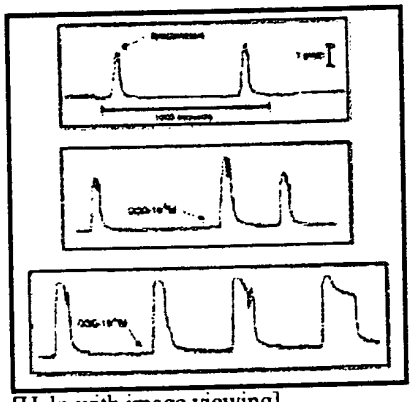

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Figure 1. Effect of cocaine (COC) on spontaneous contractile activity of term pregnant human myometrium. Arrows, Addition of 10 sup -5 and 10 sup -4 mol/L cocaine

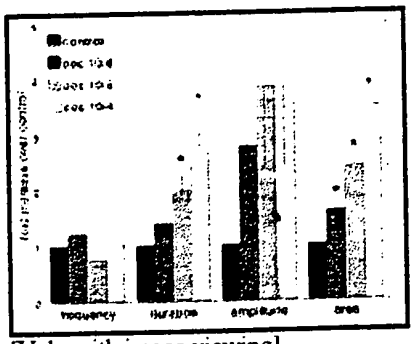

Figure 2. Effect of cocaine (COC) on contractile activity of myometrium isolated from term pregnant women (n = 5) at cesarean section. Data (mean +-\SE) expressed relative to control. Asterisk, $p < 0.001$

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Comment

To our knowledge, this is the first report of an acute effect of cocaine on the spontaneous myometrial activity in isolated pregnant human myometrium. The data indicate that the primary response is an immediate increase in duration of contractions and an increase in integrated force of contractions over time.

The lower limit of the dose range of cocaine (10 sup -6 mol/L or approximately 1000 ng/ml) was selected on the basis of previous studies that have shown peak plasma levels of 3 x 10 sup -6 mol/L in male volunteers after intravenous injection of cocaine [8]. No controlled data exist on pregnant or nonpregnant females.

The only data on peak plasma cocaine levels in pregnancy have been obtained from animal studies. In the pregnant mouse peak maternal plasma levels after intraperitoneal injection of cocaine were similar to those in humans (10 sup -6 mol/L). Peak myometrial levels, however, were nearly 100-fold greater, approaching 10 sup -4 mol/L [9]. We therefore used 10 sup -4 mol/L cocaine as the upper limit of our experimental dose range, recognizing that at concentrations higher than this cocaine may exhibit some nonspecific membrane effects.

Our data suggest that the increase in preterm labor and delivery among cocaine users may in part be explained by a direct effect on myometrial contractile activity. Although cocaine is known to inhibit catecholamine uptake and augment alpha-adrenergic pathways, in the rat the acute effect of cocaine on myometrial contractile activity was not affected by alpha-adrenergic blockade [10]. Therefore the effect of cocaine on myometrial contractile activity may be mediated by mechanisms other than potentiation of adrenergic pathways.

Hertelendy and Molnar [11] and Molnar et al [12] reported the inhibition of cyclic adenosine 2'-monophosphate generation and stimulation of inositol phosphate generation by cocaine in nonpregnant human cultured myometrial cells. Hurd et al [13] have demonstrated the down-regulation of beta-adrenergic receptors by cocaine in cultured myometrial cells of sheep after exposure to cocaine. These effects were maximal after prolonged

exposure (1.5 hours to 4 days). Whether these effects are responsible for the acute response to cocaine observed in our study remains to be elucidated.

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Cocaine Acutely Increases Rat Myometrial Contractile Activity by Mechanisms Other Than Potentiation of Adrenergic Pathways

[Papers Of The Society For Gynecologic Investigation]

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Outline

Graphics

Abstract

OBJECTIVE: We hypothesized that cocaine acutely increases contractile activity in isolated rat myometrium and that this effect is solely caused by potentiation of adrenergic pathways.

STUDY DESIGN: Isometric contractions were measured in myometrium isolated from virgin and day-18 pregnant Sprague-Dawley rats. Frequency, duration, amplitude, and integrated area were compared before and after the addition of cocaine (10 sup -6 to 10 sup -4 mol/L) by means of analysis of variance and Duncan's multiple-range test. The effects of alpha-adrenergic receptor antagonists (prazosin 10 sup -6 mol/L and yohimbine 10 sup -6 mol/L) and beta-adrenergic receptor antagonist (DL-propranolol 2 x 10 sup -6 mol/L) were

assessed.

RESULTS: Contraction duration, expressed relative to control, increased acutely after cocaine (10 sup -5 mol/L) administration in pregnant (1.70 +/-0.20) and nonpregnant (1.36 +/-0.24) myometrium (mean +/-SE, $p < 0.05$), as did integrated area (pregnant 3.47 +/-0.97, nonpregnant 2.48 +/-0.66) (mean +/-SE, $p < 0.05$). These effects were not completely inhibited by adrenergic blockade.

CONCLUSION: Cocaine acutely increases the duration and integrated area of spontaneous contractions in isolated rat myometrium by mechanisms not completely explained by inhibition of catecholamine reuptake and potentiation of adrenergic pathways. (AM J OBSTET GYNECOL 1993;169:1502-6.)

Key words: Cocaine, myometrium, rat, contractions, preterm labor

It is estimated that 30 million Americans have used cocaine. Five million Americans use cocaine regularly, and 15% of cocaine users are women of reproductive age [1]. Although cocaine use has been associated with several adverse perinatal effects, including an increase in small-for-gestational-age infants, pregnancy-induced hypertension, abruptio placentae, and congenital anomalies, perhaps the most significant impact has been an increase in preterm labor and delivery [2]. The incidence of preterm delivery among women who use cocaine has been reported to be 17% to 50% [2,3,4,5]; however, the mechanisms by which preterm labor occurs in this setting are not well understood.

There are conflicting data regarding the acute effect of cocaine on myometrial contractility. Cocaine was reported to produce tetanic contractions in myometrium isolated from the nonpregnant rabbit and the pregnant cat; however, cocaine had no effect on myometrium isolated from the nonpregnant rat [6]. Hurd et al [7] demonstrated that cocaine alone did not augment contractions but increased the contractile response of pregnant rabbit myometrium to epinephrine.

Because previous studies have varied with respect to temperature, dose of cocaine, duration of exposure, experimental animals, and method of analysis used, this study was performed to systematically examine the acute effect of cocaine on myometrium isolated from the pregnant and nonpregnant rat. The rat was chosen as the experimental model, given the volume of information published on rat myometrial physiologic and biochemical characteristics and because the short duration of rat gestation makes it convenient for studies of the pregnant state.

Material and methods

Materials. Estradiol benzoate, cocaine hydrochloride, reserpine, DL-propranolol, yohimbine, and prazosin were obtained from Sigma (St. Louis). All drugs were dissolved in sterile water, except estradiol benzoate, which was dissolved in ethanol and suspended in sesame oil.

Measurement of contractile activity. Day 18, timed, pregnant Sprague-Dawley rats and nonpregnant 175 to 199 gm Sprague-Dawley rats were cared for under the guidelines of the University of Texas at Houston Animal Care Center. Estradiol (50 micrograms/day in sesame oil) was administered subcutaneously for 2 days before removal of the uterus from the nonpregnant rats. For experiments in four nonpregnant animals reserpine (1 mg/kg/day) was administered intraperitoneally for 5 days. Animals were killed by cervical subluxation. The uteri were trimmed of excess fat and connective tissue and slit longitudinally, and the uterine cavity was gently scraped to remove most of the endometrium. Four myometrial strips were obtained from each animal. In pregnant animals these were obtained away from the implantation site. Longitudinal strips (100 mg) were incubated in buffer (composition in millimoles per liter: sodium chloride 125, potassium chloride 2.4, calcium chloride 1.8, magnesium chloride 0.5, sodium bicarbonate 23.9, and glucose 11.0) in jacketed tissue baths aerated with 95% oxygen and 5% carbon dioxide at 37 degrees C. The uterine strips were suspended at 1 gm tension for 20 to 30 minutes before the addition of the experimental drugs. Isometric contractions were monitored by force-displacement transducers (Grass FTO3, Grass, Quincy, Mass.) connected to a Beckman R411 Dynograph recorder (Beckman, Palo Alto, Calif.). The recorder paper speed was set at 0.1 mm/sec and calibrated so that 1 cm of vertical displacement represented 1 gm of tension.

Data analysis. The characteristics of the contractions analyzed over 1000-second intervals immediately before and after the addition of drugs included frequency (number per 1000 seconds), mean duration (seconds) and amplitude (grams) of each contraction, and integrated area under the contraction curve (representing contractile force over 1000 seconds) measured with a digitized plotter. Data are reported as mean +/-SE and were analyzed by analysis of variance of log-transformed data and Duncan's multiple-range test.

Results

Acute effects of cocaine on contractile activity. Cocaine exposure acutely increased contractile activity in myometrial strips isolated from the nonpregnant rat (n = 4 rats) Figure 1, with the greatest effect on the duration of contractions and the integrated area Figure 2. The effects were concentration dependent, reaching statistical significance at concentrations of 10 sup -5 and 10 sup -4 mol/L. The frequency of contractions decreased with increasing concentrations of cocaine, reaching statistical significance at a concentration of 10 sup -4 mol/L. Because of variability in the frequency and amplitude of spontaneous contractions between animals, the data are expressed relative to control.

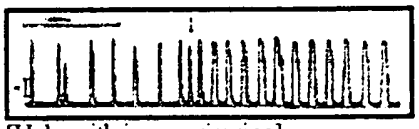

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Figure 1. Effect of 10 sup -5 mol/L cocaine on spontaneous contractile activity of myometrium isolated form virgin Sprague-Dawley rat. Arrow, Point of addition of cocaine

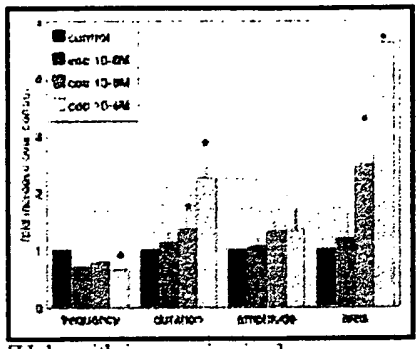

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Figure 2. Effect of cocaine (COC) on contractile activity of myometrium isolated from virgin Sprague-Dawley rats (n = 4). Data (mean +/-SE) expressed relative to control (asterisk, $p < 0.05$). Spontaneous contraction parameters (control): frequency 14.5 per 1000 seconds, duration 25.3 seconds per contraction, amplitude 3.2 gm per contraction, area 260 gm sec (measured over 1000 seconds)

Cocaine had similar effects on contractions in myometrium isolated from day 18 pregnant rats (n = 5 rats) (Figs. 3 and 4). Duration and integrated area of contractions increased significantly after acute exposure to 10 sup -5 and 10 sup -4 mol/L cocaine. There was an increase in contraction amplitude, which reached statistical significance at 10 sup -4 mol/L. The frequency of spontaneous contractions was lower in pregnant than in nonpregnant animals (8.3 +/-4.3 vs 14.3 +/-1.9 contractions per 1000 seconds). Although there was a trend toward increasing frequency of contractions in myometrium from pregnant animals in response to cocaine, no statistically significant difference was observed.

Role of catecholamines in the effect of cocaine. Cocaine has been shown to inhibit catecholamine reuptake [8] and to augment the contractile response to epinephrine in the pregnant rabbit [7]. To assess whether cocaine affected rat myometrial activity through augmentation of alpha-adrenergic pathways, the alpha-receptor blocking agents yohimbine (10 sup -6 mol/L) and prazosin (10 sup -6 mol/L) were added to the baths 30 minutes before the addition of cocaine (n = 6 nonpregnant rats). alpha-Adrenergic receptor blockade by these agents was confirmed by their ability to block the contractile response to methoxamine. Methoxamine (10 sup -5 mol/L) acutely increased the frequency, duration, and integrated area of contractions in myometrium from nonpregnant rats; these effects were inhibited by pretreatment with yohimbine and prazosin Figure 5. In contrast, the effects of cocaine (10 sup -5 mol/L) on contraction duration and integrated area were not inhibited by the same concentration of these agents.

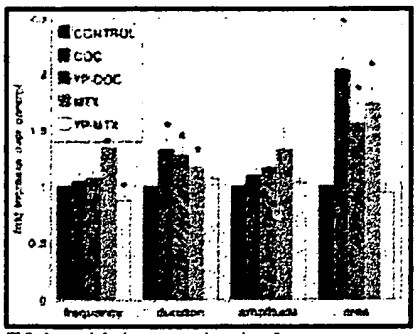

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Figure 5. Lack of complete inhibition by alpha-adrenergic blockade on effect of 10 sup -5 mol/L cocaine (COC) on contractile activity of nonpregnant rat myometrium (n = 6). Data (mean +/-SE) expressed relative to control (asterisk, $p < 0.05$). YP-COC, Pretreatment with prazosin (10 sup -6 mol/L) and yohimbine (10 sup -6 mol/L) 30 minutes before addition of cocaine; MTX, effect of methoxamine (10 sup -5 mol/L) on spontaneous activity; YP-MTX, effect of pretreatment with prazosin (10 sup -6 mol/L) and yohimbine (10 sup -6 mol/L) before addition of methoxamine (10 sup -5 mol/L)

Previously, propranolol (D- and DL-) had been shown to diminish the response of isolated rabbit myometrium to cocaine [7]. Addition of DL-propranolol (2 x 10 sup -6 mol/L) to the bath did not alter the effect of cocaine on rat myometrial contractile activity (data not shown).

Four nonpregnant rats were pretreated with reserpine to deplete catecholamine stores, to explore the role of catecholamines in the contractile response of rat myometrium to cocaine. As noted previously, [8] there was an increase in spontaneous contractile frequency of myometrium isolated from rats pretreated with reserpine (29.7 per 1000 seconds) compared with that of untreated controls (14.5 per 1000 seconds). Cocaine (10 sup -5 mol/L) caused no further increase in contractile activity in myometrium from animals pretreated with reserpine (data not shown).

Comment

To our knowledge, this is the first report of an acute effect of cocaine on spontaneous in vitro rat myometrial contractile activity. The data indicate that cocaine augments spontaneous contractile activity in myometrium from both the nonpregnant and 18 day (near term) timed pregnant rat. The primary response to cocaine was an increase in duration of contractions and an increase in integrated force over time. These effects reached statistical significance at a cocaine concentration of 10 sup -5 mol/L but demonstrated similar trends at 10 sup -6 mol/L. The peak plasma concentration of cocaine after intravenous administration or inhalation of the alkaloid-base "crack" cocaine is estimated at 3 x 10 sup -6 mol/L [9]. Pharmacokinetic studies of cocaine in the pregnant mouse have shown peak myometrial tissue levels of cocaine to be 100,000 micrograms/gm tissue or almost 100-fold greater than peak maternal plasma levels 30 minutes after intravenous administration [10]. Cocaine concentrations of 10 sup -5 and 10 sup -4 mol/L may have clinical relevance at the myometrial level; however, pharmacokinetic studies in the pregnant human are lacking.

Cocaine had previously been shown to augment contractile response to epinephrine in pregnant rabbit myometrium [7]. In that study the ability of

cocaine to augment epinephrine-induced myometrial contractility was inhibited by both D- and DL-propranolol (2 x 10 sup -6 mol/L). DL-Propranolol is a beta-receptor blocker. The stereoisomer D-propranolol has no beta-blocking activity but retains membrane stabilizing properties. The authors suggested that cocaine may alter membrane stabilization [7]. We did not find that DL-propranolol modified the contractile response to cocaine in rat myometrium. This difference may be explained by the use of a different animal model in which cocaine exhibits an acute effect on spontaneous rat myometrial contractile activity that is not simply due to membrane destabilization.

Cocaine inhibits catecholamine reuptake and increases circulating catecholamines. It has been suggested that this action might be the mechanism by which cocaine increases myometrial contractile activity [1]; however, previous work has demonstrated that there are few functioning adrenergic nerve endings in the term human uterus [11]. Furthermore, increased circulating catecholamines have been shown to increase incoordinate activity of the uterus but not to decrease the duration of labor [12]. Finally, alpha-adrenergic blockade with yohimbine and prazosin, at doses previously shown [13] and confirmed in this study to block the methoxamine-stimulated contractile response, did not inhibit the myometrial response to cocaine. These data suggest that facilitation of adrenergic pathways may not be the sole mechanism by which cocaine acutely increases rat myometrial contractile activity.

Reserpine has been shown to deplete catecholamine stores and to increase spontaneous contractile activity of rat myometrium, possibly by removal of tonic beta-adrenergic inhibition [8]. In tissue from reserpine-treated rats in our study cocaine did not increase contractile activity further. These results suggest that in this model catecholamine depletion results in loss of the effect of cocaine. Although this may seem to contradict the data obtained from pretreatment with adrenergic blockers, it should be noted that reserpine depletes catecholamines that act on both alpha- and beta-adrenergic receptors. In contrast, yohimbine and prazosin selectively block alpha-adrenergic receptors. Therefore, in addition to decreasing alpha-adrenergic influence, reserpine also diminishes beta-adrenergic influence, which may be important in the tonic inhibition of spontaneous myometrial activity. Myometrium from reserpine-pretreated rats may therefore be exhibiting enhanced spontaneous phasic contractile activity, making the response to acute cocaine exposure less evident.

Our data suggest that the increase in preterm labor and delivery among cocaine users could in part be explained by a direct effect of the drug on myometrial contractile activity. Recently, Hertelendy and Molnar [14] and Molnar et al [15] described inhibition of cyclic adenosine 2'-monophosphate generation and stimulation of inositol phosphate generation in cultured human myometrial cells by cocaine. In addition, Hurd et al [16] have demonstrated a down-regulation of beta-adrenergic receptors in sheep cultured myometrial cells after exposure to cocaine. The maximum effect of

cocaine in each of these studies was obtained over prolonged exposure (1.5 hours to 4 days). The mechanism of the cocaine-induced acute increase in myometrial contractile activity shown in our study may not reflect these reported actions and remains to be elucidated.

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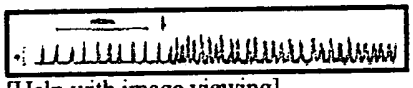

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Figure 3. Effect of 10 sup -4 mol/L cocaine on spontaneous contractile activity of myometrium isolated from day 18 pregnant Sprague-Dawley rat. Arrow, Point of addition of cocaine

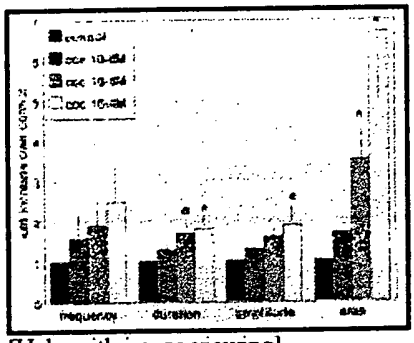

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Figure 4. Effect of cocaine (COC) on contractile activity of myometrium isolated from day 18 pregnant Sprague-Dawley rats (n = 5). Data (mean +/-SE) expressed relative to control (asterisk, $p < 0.05$). Spontaneous contraction parameters (control): frequency 8.6 per 1000 seconds, duration 25.0 seconds per contraction, amplitude 2.3 gm per contraction, area 127 gm sec (measured over 1000 seconds)

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Cocaine Alters Placental Production of Thromboxane And Prostacyclin

[Transactions Of The Fourteenth Annual Meeting Of The Society Of Perinatal Obstetricians]

Monga, Manju; Chmielowiec, Susie; Andres, Robert L.; Troyer, Lisa R.; Parisi, Valerie M.

Outline

Graphics

Abstract

OBJECTIVE: The objective of this study was to test the hypothesis that cocaine alters placental prostaglandin production in vitro.

STUDY DESIGN: Placentas were obtained from healthy women (n = 6) after normal vaginal delivery at term. Placental explants (300 mg) were incubated in duplicate at 37 degrees C in the presence of 0, 30, 300, or 3000 ng/ml cocaine. Thromboxane and prostacyclin production was measured by radioimmunoassay of their stable metabolites (thromboxane B_2 and 6-keto-prostaglandin F_{1alpha}) at 0, 0.5, 1.0, 1.5, 2, 4, 8, and 12 hours. Analysis of variance with Newman-Keuls test was used for statistical analysis.

RESULTS: Cocaine increased thromboxane production in a dose-dependent manner ($p < 0.001$) and decreased prostacyclin production ($p < 0.05$). Cocaine increased the ratio of thromboxane/prostacyclin production ($p < 0.05$).

CONCLUSION: Cocaine alters the placental production of prostaglandins in vitro, favoring thromboxane production, which may cause vasoconstriction and decrease uteroplacental blood flow. (AM J OBSTET GYNECOL 1994;171:965-9.)

Key words: Cocaine, placenta, prostaglandins

It is estimated that 30 million Americans have used cocaine at least once and that 5 million use the drug regularly [1]. Cocaine use has increased in women of reproductive age. Fifteen percent of the total number of regular cocaine users are women between the ages of 18 and 34, and the reported prevalence of cocaine use in pregnancy is as high as 10% [2,3].

Even after confounding variables such as age, race, smoking, and polydrug use are controlled for, the use of cocaine in pregnancy is associated with an increased incidence of preterm labor, intrauterine growth restriction, abruptio placentae, and pregnancy-induced hypertension [4,5,6,7,8]. These complications have been attributed to short-term effects of cocaine, including inhibition of catecholamine reuptake, [9] vasoconstriction of uterine and umbilical vessels, [10] and direct augmentation of myometrial contractile activity [11,12].

Many adverse perinatal outcomes associated with cocaine use are also observed in pregnancies complicated by preeclampsia, antiphospholipid syndrome, and class RF diabetes. In these conditions placental production of thromboxane A_2 (TXA_2) and prostacyclin (PGI_2) is altered, [13,14,15,16] with an increase in the ratio of TXA_2 to PGI_2. This may result in vasoconstriction, platelet aggregation, and decreased uteroplacental blood flow, which are characteristic of these conditions [17]. The objective of this study was to determine the in vitro effect of cocaine on placental production of TXA_2 and PGI_2.

Material and methods

Placentas were obtained from six healthy women immediately after normal vaginal delivery at term. Pregnancies complicated by intrauterine growth restriction, hypertension, diabetes, or antiphospholipid syndrome were excluded. The patients were nonsmokers and denied substance abuse or the use of prostaglandin inhibitors during pregnancy. This investigation was approved by the Committee for the Protection of Human Subjects at the University of Texas at Houston.

Under sterile conditions the placenta was washed repeatedly with Dulbecco's modified Eagle medium (Gibco, Grand Island, N.Y.). The basal plate of the placenta was removed and discarded, and multiple placental explants were dissected, minced, and washed with Dulbecco's modified Eagle medium. Approximately 300 mg of placental tissue was placed in individual incubation wells, each containing 9 ml Dulbecco's modified Eagle medium and 0, 30, 300, or 3000 ng/ml cocaine hydrochloride (Sigma Chemical Co., St. Louis). The wells without cocaine served as controls. In addition, four wells without tissue were filled with 9 ml Dulbecco's modified Eagle medium and 0 to 3000 ng/ml cocaine hydrochloride to

confirm that no prostaglandins are produced in the absence of placental tissue.

The wells were incubated in duplicate for 12 hours under sterile conditions at 37 degrees C in a Dubnoff shaker and oxygenated with 95% oxygen and 5% carbon dioxide. This technique has been previously validated [16]. At 0, 0.5, 1, 1.5, 2, 4, 8, and 12 hours of incubation, 200 microliters aliquots of media were collected and stored at -20 degrees C. Placental TXA_2 and PGI_2 production was determined by radioimmunoassay of their stable metabolites, thromboxane B_2 (TXB_2) and 6-keto-prostaglandin F_{1alpha} (6-keto-PGF_{1alpha}), respectively. The radioimmunoassays involved competitive binding of radioactive and natural TXB_2 (or 6-keto-PGF_{1alpha}) to a highly specific antibody. The antibodies, standards, and tracers were obtained from Advanced Magnetics (Cambridge, Mass.). Equal amounts of antibody, tracer, and sample (or known standard) were incubated at 4 degrees C for approximately 20 hours. Cold dextran-coated charcoal was then added, and the samples were spun at 2000g at 4 degrees C for 20 minutes. The supernatant was extracted, added to 15 ml of scintillation fluid, and counted on a Beckman Gamma Counter for 10 minutes per sample. A standard curve was created by plotting known standards as a percentage of the zero dose tubes. Sample concentrations were calculated from the standard curve by percent binding obtained in the assay for each individual sample. Serial sample dilutions were parallel to the standard curve ($p > 0.5$). Samples containing Dulbecco's modified Eagle medium plus 0 to 3000 ng/ml cocaine without placental tissue resulted in a zero dose response. The least detectable concentrations were 1.5 +/-0.33 pg for TXB_2 and 2.45 +/-0.57 pg for 6-keto-PGF_{1alpha}. Intraassay and interassay variation was <=7.8% and <=4.6%, respectively, for TXB_2, and <=8.0% and <=6.2%, respectively, for 6-keto-PGF_{1alpha}.

Data are expressed in picograms per milligram of wet tissue as the mean +/-SE. Statistical differences in prostaglandin production were analyzed with analysis of variance with post hoc analysis by means of Student--Newman-Keuls test, and $p < 0.05$ was chosen to represent statistical significance.

Results

The effect of cocaine on TXB_2 and 6-keto-PGF_{1alpha} production is summarized in Table I. Cocaine increased placental TXB_2 production as compared with control in a dose-dependent manner as shown in Fig. 1. This was significant ($p < 0.001$) with each concentration of cocaine differing significantly from control and from each other at each time point studied. As shown in Fig. 2, cocaine decreased placental 6-keto-PGF_{1alpha} production as compared with control at each concentration tested ($p < 0.05$). Each concentration of cocaine differed significantly from control but not from

each other at each time point studied. The rate of prostaglandin production was significantly different from control at each concentration of cocaine ($p < 0.05$). These data are shown in Table II. Cocaine at 30, 300, and 3000 ng/ml increased the ratio of TXB_2 production/6-keto-PGF_{1alpha} production as compared with control ($p < 0.05$). This effect decreased with increased duration of incubation (data shown for cocaine concentration of 300 ng/ml in Fig. 3).

Comment

Cocaine use during pregnancy is associated with an increase in adverse perinatal outcome similar to that observed in pregnancies complicated by preeclampsia, antiphospholipid antibody syndrome, and class RF diabetes [3,4,5,6,7]. In the latter conditions placental prostaglandin production is altered [13,14,15,16]. Therefore we hypothesized that cocaine alters placental production of TXA_2 and PGI_2. We found that in vitro exposure of placental explants to cocaine significantly increased TXA_2 production, decreased PGI_2 production, and increased the TXA_2/PGI sub 2 ratio.

Previously, cocaine (10 to 1000 micrograms/ml) was shown to decrease prostacyclin production by human cultured umbilical arterial endothelial cells after 24 hours of exposure [18]. To our knowledge our investigation is the first to examine the effect of cocaine on the production of prostaglandins by cultured placental tissue. We chose the concentrations of cocaine on the basis of peak plasma cocaine levels of 300 to 1000 ng/ml after intravenous or intranasal administration of cocaine by male volunteers [19,20]. We are unaware of any controlled data regarding cocaine levels in pregnant or nonpregnant women.

In previous studies of prostaglandin production in the human placental explant model, incubation was continued for 48 hours. In this study the duration of incubation was limited to 12 hours because the half-life of cocaine in humans is 40 to 80 minutes [20]. It has been shown after short-term administration of cocaine that plasma levels are minimal after 12 hours [19,20].

We found that cocaine increased the ratio of TXA_2 to PGI_2 but that this effect decreased with duration of incubation. Cocaine is metabolized to benzoylecgonine, norcocaine, and ecgonine methyl ester [20]. Roe et al [21] showed that in placental microsome culture the concentration of cocaine decreased by 20% after 135 minutes of incubation. They concluded that placental cholinesterase metabolizes cocaine to ecgonine methyl ester. It is not known whether cocaine levels decline in our placental culture model with time, or if decreasing concentrations correlate with the observed pattern of thromboxane/prostacyclin production.

Cocaine acutely causes vasoconstriction of umbilical and uterine blood

vessels [9]. An increase in the TXA_2/PGI_2 ratio may also result in placental vasoconstriction and decreased uteroplacental flow [17]. This increase in the ratio of TXA_2/PGI_2 may partially explain the association of maternal cocaine abuse with intrauterine growth restriction, abruptio placentae, spontaneous abortion, and hypertension. Cocaine acutely augments myometrial contractile activity, [11,12] but TXA_2 stimulates and PGI_2 inhibits myometrial contractile activity [22]. Therefore the observed alteration in prostaglandin metabolism may play a role in the pathophysiology of preterm labor among cocaine users.

Future studies aimed at identifying the point in the pathway of arachidonic acid metabolism affected by cocaine and the specific cell type that is responsible for these changes are necessary. This will greatly enhance our understanding of the effect of cocaine on the uteroplacental-fetal unit. Such studies may eventually lead to successful pharmacologic manipulation of placental prostaglandin production in pregnant women who use cocaine.

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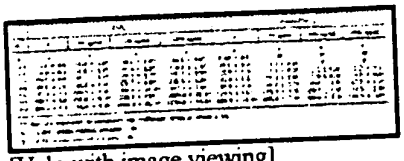

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Table I. Effects of cocaine (0 to 3000 ng/ml) on TXB_2 and 6-keto-PGF sub 1alpha production

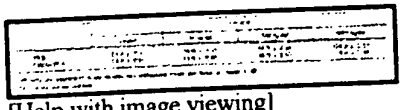

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Table II. Production rate of prostaglandins by placental tissue

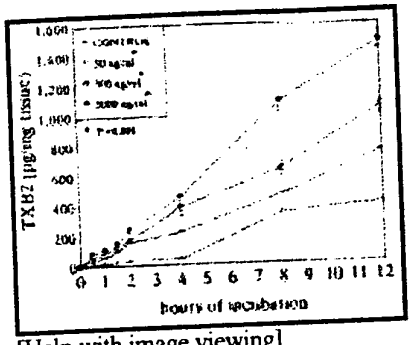

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Figure 1. In vitro effect of cocaine on placental production of TXA_2, as measured by its stable metabolite TXB_2 (picograms per milligram tissue). Data shown are mean +/-SE (n = 6). SE <10 not shown. At each time point each concentration of cocaine was significantly different from control and from each other ($p < 0.001$)

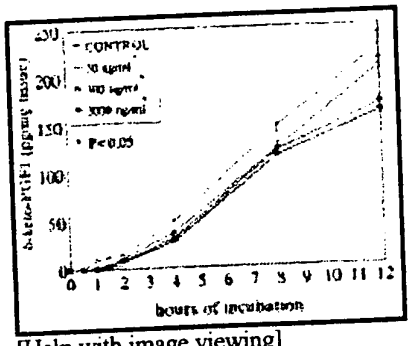

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Figure 2. In vitro effect of cocaine on placental production of PGI_2, as measured by its stable metabolite 6-keto-PGF_{1alpha} (picograms per milligram tissue). Data shown are mean +/-SE (n = 6). SE <5 not shown. At each time point each concentration of cocaine was significantly different from control ($p < 0.05$)

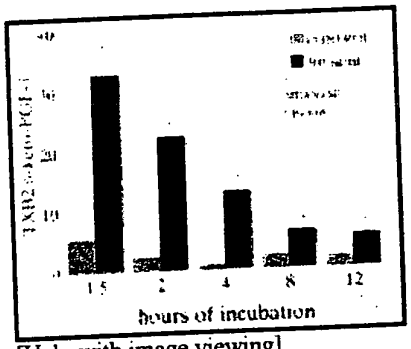

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Figure 3. In vitro effect of cocaine (300 ng/ml) on ratio of placental production of TXA_2/PGI_2. Data shown are mean +/-SE (n = 6). Asterisk, $p < 0.05$

American Journal of Obstetrics and Gynecology

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Comparison of P rceived and Actual Rates of Survival and Freedom From Handicap in Premature Infants

[General Obstetrics And Gynecology]

Haywood, James L.; Goldenberg, Robert L.; Bronstein, Janet; Nelson, Kathleen G.; Carlo, Waldemar A.

Outline

Graphics

Abstract

OBJECTIVE: Our goal was to learn whether physicians delivering obstetric care accurately estimated rates of survival and freedom from handicap in premature infants.

STUDY DESIGN: We surveyed by mail 409 obstetricians and general and family physicians reported to perform deliveries in Alabama to identify their perceptions regarding survival and handicap-free rates of infants born at gestational ages between 23 and 36 weeks, inclusive. Responses were compared with published national rates of survival and freedom from handicap by means of unpaired t tests.

RESULTS: A total of 224 physicians responded (55%), and 183 were still practicing obstetrics. They significantly underestimated survival rates from 23 through 34 weeks' gestation ($p < 0.05$) and freedom from serious handicap from 23 through 36 weeks' gestation ($p < 0.05$). They advocated early treatment of preterm labor, but <50% would perform cesarean delivery for fetal distress before 26 weeks' gestation.

CONCLUSION: We conclude that physicians delivering obstetric care significantly

underestimate survival and freedom from handicap in preterm infants. Perinatal care may be adversely affected by these misperceptions. (AM J OBSTET GYNECOL 1994;171:432-9.)

Key words: Premature infant, infant mortality, neonatal mortality, neonatal handicap, neonatal survival

For more than a decade there has been concern that misperceptions about neonatal survival and eventual quality of life by physicians who deliver babies may result in less than appropriate care for pregnant women and prematurely born infants. In 1982 Goldenberg et al [1] surveyed Alabama physicians who delivered babies to determine their perceptions about the survival of preterm infants. They reported that physicians underestimated potential for survival in preterm infants. Additionally, the study showed, through management of hypothetical cases, that treatment decisions based on inaccurate knowledge would result in potentially viable fetuses receiving less than optimal management [1]. This study was performed at a time when survival rates for preterm infants were only 50% at 28 weeks' gestation and 90% at 32 weeks' gestation. A similar study, conducted by Trudinger and Boshell, [2] reported in 1985 that Australian obstetricians were inappropriately pessimistic concerning neonatal survival and were uncertain about proper use of tocolytics and prenatal steroids. During the intervening years survival has improved to approximately 50% at 25 weeks and >80% at 28 weeks, without increased rates of serious handicap [3,4,5].

In a recent preliminary report, Sanders et al [6] described the results of their study in which they surveyed a national sample of neonatologists to gauge their perceptions regarding survival of preterm infants. A total of 463 of 600 respondents (77%) believed infants born before 25 weeks were potentially viable, whereas 506 (84%) believed their obstetric colleagues considered infants born at <=24 weeks to be potentially viable. Most indicated that they would resuscitate at >=24 weeks' gestation, although expecting a 50% mortality at 24 weeks.

Reduction in infant mortality is occurring with increasing survival of preterm infants, but many question the advisability of resuscitating or aggressively treating extremely immature neonates who carry relatively high rates of morbidity if they do survive. Wood et al [7] studied a group of infants born from 1986 through 1988 and weighing <=1600 gm at birth. They reported that combined handicap rates at hospital discharge, defined as bronchopulmonary dysplasia or intraventricular hemorrhage grade III or IV, were 100% at 24 weeks, 56% at 25 weeks, and 46% at 26 weeks. Beyond 26 weeks handicap rates declined rapidly. Recently, Robertson et al [4] reported on a large group of Canadian infants with birth weights of 500 to 1250 gm. In this cohort study survival improved from 67% in 1978 and 1979 to 86% in 1988 and 1989, with greatest improvement seen in the smaller infants. Children were considered disabled if they had cerebral palsy, neurosensory hearing loss, visual acuity <20/60 in the better eye, convulsive disorder, or developmental mental index or intelligence quotient >3 SD below the mean

on standardized testing at 1 year of age. Early childhood disability rates for survivors were not increased, despite the noted improvement in survival. Disability rates in the later cohort were 26% for the 500 to 749 gm group, 13% for the 750 to 799 gm group, and 14% for the 1000 to 1250 gm group.

It is known that survival rates of preterm infants improve proportionally to increased transfer rates of those infants to perinatal centers and that survival is further enhanced when transfer is accomplished prenatally [8,9]. Despite an increase in the proportion of preterm infants transferred in utero to perinatal centers, there may be a sizable proportion of preterm infants born in hospitals without advanced perinatal services [10].

With the evolution of perinatal care and its potential impact on infant mortality, we questioned how the level of optimism about survival and severe handicap-free states correlated with the level of perinatal care. To test the hypothesis that physicians underestimate survival and freedom from handicap of preterm infants and to further understand the relationship between perceptions of outcome and physicians' propensity to intervene in preterm labor, we surveyed Alabama physicians who deliver babies.

Methods

A questionnaire was designed to assess the perceptions of physicians who deliver babies about survival rates of preterm infants born at gestational ages from 23 through 36 weeks. The survey instrument was quite similar in form to the one we used in 1982 [1]. Participants were asked to estimate for each completed week of gestational age the percentage of infants who would be expected to survive if born in a perinatal center. Additionally, they were asked to estimate the percent of survivors at each completed week of gestational age who would be free of major handicap. Because estimation of fetal weight is fraught with pitfalls, we chose estimated gestational age as the most relevant datum available to the obstetrician when making decisions regarding intervention in preterm labor.

Through management of a hypothetical case of a healthy primigravid woman in preterm labor with intact fetal membranes, we also asked (1) what interventions they would make at each week of gestation, including whether they would administer steroids or tocolytics, monitor the fetus in labor, perform cesarean section for fetal distress or breech presentation, or perform cesarean delivery for a vertex-presenting fetus who was not in distress and (2) whether they would transport the patient to a perinatal center capable of ventilating sick newborns (assuming their own hospital did not have that capability).

In addition, we requested demographic information, including age, gender, year of graduation from medical school, and last year of any formal training. We inquired about both the field of training (obstetrics, family or general medicine, or other) and the level of training (internship with or without residency). This new survey was administered to a group of obstetricians

and house officers at our hospital to test for clarity and modified accordingly.

Cover letters and questionnaires were mailed to all physicians believed to be delivering babies in Alabama. The respondents were given the option of identifying themselves. A second mailing was directed to all those who had not identified themselves in a response to the first mailing. Participants were asked to discard the second questionnaire if they had responded to the first request. Data were entered into a commercial computer spreadsheet program (Excel for Windows, version 4.0; Microsoft Corp., Redmond, Wash., 1992) and statistical analysis was performed with the program's Analysis ToolPak option.

We used data obtained from the 1988 and 1989 National Institute of Child Health and Human Development (NICHHD) Neonatal Network [3] and from the 1982 to 1986 March of Dimes Multicenter Prevention Trial [11] as our standards for actual survival rates. NICHHD data were chosen as standards for survival at gestational ages of 23 through 30 weeks inclusive, because they are the most recent national data published regarding survival of extremely premature infants. However, because this was a study designed to measure outcomes of infants who were born at birth weights of <1500 gm and admitted to newborn intensive care units, infants of later gestational ages were more likely to have been small for gestational age (SGA). Premature infants who are also SGA are more likely to have poor outcomes; therefore we derived standard survival rates for infants of 31 through 36 weeks' gestation from the March of Dimes trial, which entered all births at <37 weeks' gestation, regardless of birth weight or admission to a newborn intensive care unit.

To further analyze survival data we developed confidence intervals and compared them to respondents' estimates. The NICHHD report grouped 1765 infants in ranges of <=23 weeks (6%), 24 to 25 weeks (15%), 26 to 27 weeks (22%), and 28 to 31 weeks (44%) according to obstetric measures. From these proportions the number of infants in each gestational age range was evenly divided by the number of weeks in the range to estimate the number of infants assigned to each completed week of gestational age. All surviving infants in the NICHHD Network's <=23 weeks' gestation group were assumed to be 23 weeks' gestation for the purposes of this analysis. The number of infants born at each week, 31 through 36, was known for the March of Dimes study. With the use of reported survival means and the number of infants assigned to each gestational age, 95% confidence intervals were constructed for actual survival at each week. The lower confidence limit for each week of gestation, 23 through 36, was compared with the respondents' mean estimate by means of an unpaired t test, thus allowing for variability among reporting centers as a result of differences in sample size and allowing the assumption of a normal distribution.

We defined handicap-free rates from a meta-analysis of outcome studies published between 1978 and 1984 [12]. These defined major handicaps as

cerebral palsy, mental retardation (development or intelligence quotients <70), blindness, deafness, or generalized severe failure to thrive. This reference was chosen because handicap rates were reported by gestational age rather than birth weight. The estimates of freedom from handicap at each gestational age were compared to actual rates with the use of unpaired t tests performed at each gestational age between 23 and 36 weeks' gestation inclusive.

Results

Two hundred twenty-four of 409 physicians (55%) responded to the two mailings, with 183 indicating they still practiced obstetrics. Demographic variables did not differ among obstetricians (n = 155) and general and family physicians (generalists n = 28), except that the obstetrician group had more male physicians (90%) than the nonobstetrician group (79%, $p < 0.05$). Characteristics of respondents are summarized in Table I. Those who responded to the second mailing, which was geographically coded, were geographically representative of the eligible population. One respondent gave nonsensical estimates (very high survival estimates at low gestational ages and low estimates at more advanced gestational ages). This anonymous respondent's answers were eliminated from analysis.

Participants significantly underestimated survival rates at each week between 23 and 34 weeks' gestation inclusive ($p < 0.05$) Figure 1. There was a significant difference among obstetricians and generalists in their responses, with generalists underestimating survival to a greater degree at 23 and 28 through 34 weeks of gestation ($p < 0.05$ for all comparisons). Responses regarding survival rates were not affected by other demographic characteristics. Although national survival rates at perinatal centers now approximate 20% at 23 weeks and 50% at 25 weeks and are >80% at 27 weeks, the combined group of participants markedly underestimated these survival rates to be 5%, 22%, and 51%, respectively.

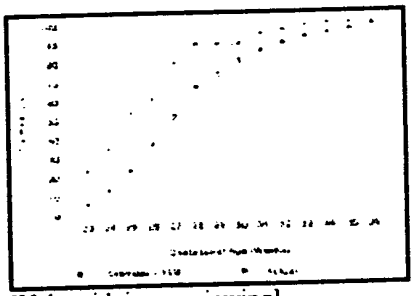

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Figure 1. Comparison of actual versus estimates of survival for infants of 23 to 36 weeks' gestation. Respondents' underestimations of survival rates were significant ($p < 0.05$) from 23 through 34 weeks' gestation

The comparison between participants' estimations and published handicap-free rates of infants born at 23 through 36 weeks' gestation is shown in Fig. 2. Differences were statistically significant at each gestational age ($p < 0.05$ for all comparisons). Generalists tended to further

underestimate handicap-free rates from 28 through 31 weeks ($p < 0.05$ for all comparisons). Again, results were not affected by other demographic characteristics. There was a wide variability among participants' estimations of survival and freedom from handicap, particularly at earlier gestational ages (Figs. 3 and 4).

Regarding interventions for preterm labor, participants indicated that on average they would first administer tocolytics to inhibit labor at 22 weeks' gestation and would both electronically monitor the fetus and transfer to a perinatal center for management of preterm labor at a mean of 23 weeks' gestation. Ninety-four percent of the participants would administer steroids to enhance fetal maturity at some gestational age, doing so at a median earliest gestational age of 25 weeks. Only half of the respondents would have intervened with cesarean delivery for fetal distress at 25 weeks' gestation, when the survival rate is known to be >50%. Fig. 5 illustrates the variation in physicians' beliefs about survival, showing the earliest gestational age at which physicians would intervene with cesarean delivery for fetal distress. Half would intervene with operative delivery at 25 weeks, but 10% would not have intervened with cesarean section for fetal distress even as late as 27 weeks' gestation.

Comment

The rationale for this study was to learn, 10 years after surveying our population of physicians who deliver babies, whether they now have an accurate knowledge of survival and outcome of premature infants, especially those born at earlier gestational ages whose outcome expectations have improved markedly since the last survey. We also sought to learn whether physicians who had pessimistic expectations of outcome would remain unwilling to intervene with aggressive management of preterm labor. Our study demonstrates that physicians performing deliveries in the targeted area significantly underestimate survival and handicap-free rates for premature infants.

The survey technique used in this study was very similar to that used by Goldenberg et al [1] in 1982, so a substantial portion of it had been pretested. More than 50% of our targeted physicians completed and returned the questionnaire. Even so, the accuracy of a survey to assess knowledge and perceptions is limited by several factors, including the ability to reach the appropriate target audience and the ability of the individual respondent to interpret the question as it is asked and answer appropriately [13]. A respondent's answer to one question may be dependent on his or her answer to another. Study participants may say what they believe is correct even though it may not agree with what they do in practice.

When our studies of 1982 and 1992 are compared, it is clear that there has been a change in physicians' estimates of survival and freedom from handicap and their willingness to intervene in early gestational age pregnancies. As an example, the gestational age at which 50% of the

physicians would have intervened by transferring the woman to a perinatal center or by performing cesarean section for fetal distress moved forward by 5 and 3 weeks, to 23 and 25 weeks, respectively, between the 1982 and 1992 studies. Despite these changes, we found that physicians who deliver babies in Alabama still underestimate both survival and freedom from handicap of premature infants at early gestational ages, especially those gestational ages at which there have been recent and rapid improvements in survival. The underestimation of survival rates at lower gestations may be due in part to the recent advances in neonatal care, including surfactant therapy for respiratory distress syndrome, which have further improved outcomes of premature infants [14]. Some attribute the recent overall decline in U.S. infant mortality to the advent of surfactant therapy [15].

Even though studies have shown the prevalence of antenatal steroid use to be highly variable and generally low, varying from 0% to 50% in some series, [16,17] 94% of our study participants indicated they would use steroids at some gestational ages to enhance fetal pulmonary maturation. Unfortunately, because the majority of questionnaires were completed anonymously, we had no way to compare respondents and nonrespondents for characteristics that might have affected results regarding this or other questions.

The obstetrician's perception of fetal viability may strongly influence decisions to intervene on behalf of the fetus. The work of Paul et al [18] showed that obstetricians tend to underestimate fetal weight, and in cases where fetal weight was underestimated at <1000 gm, neonatal mortality was significantly higher than when weight was more accurately estimated [18]. Additionally, this work, as well as that of Goldenberg et al, [1] showed that physicians' practices have appeared to reflect their knowledge, thus suggesting that women in preterm labor may not be appropriately treated if their phsyicians believe that expected survival or quality of life would be poor.

Uniform standards for the prediction and description of long-term impairment in children are somewhat elusive. As opposed to dichotomous variables such as survival, handicap rates for surviving premature infants are measured and reported according to variable criteria, such as type of impairment (sensory, motor, intellectual), age at follow-up, and testing protocol. However, most agree in broad terms that severe vision or hearing impairment, cerebral palsy, or subnormal intellect are handicapping conditions that limit quality of life and ultimate potential of children. Most studies have reported handicap rates on the basis of birth weight strata. Because we were unable to construct confidence intervals for the meta-analysis that we used as a reference for actual rates of freedom from handicap, we converted birth weights to gestational ages using approximations from a standard fetal weight --gestational age chart [19] to mimic handicap rates by gestational age from the more recent reports of Robertson et al, [4] Grogaard et al, [5] and Weissman et al [20]. Handicap rates for all three studies were lower at all gestational ages than the standard

rates used in this study.

Three very recent reports describe outcome directly in terms of gestational age at birth [21,22,23]. Whyte et al [21] found, in their series of Canadian infants of 23 through 36 weeks' gestation, outcomes similar to the standard we used for comparison, with 6 of 12 survivors born at 23 weeks, 20 of 49 at 25 weeks, and 65 of 109 at 25 weeks being cognitively normal and free of major handicaps including neurologic or sensory deficit at 2 years. In a British study Johnson et al [22] followed up 164 survivors of 23 through 27 weeks' gestation to 4 years of age. Forty percent (2 of 5 survivors) at <=24 weeks' gestation and 55% and 78% at 25 and 26 weeks' gestation, respectively, were found to be free of severe disability, defined as cerebral palsy with severely limited function, blindness, deafness, or developmental quotient <70. However, it should be noted that both studies reported much larger proportions of surviving infants with less severe impediments (minor motor deficits and cognitive delays), which are nevertheless likely to be handicapping to performance in standard school settings.

Allen et al [23] reported on a U.S. single-hospital cohort of infants of 22 to 25 weeks' gestation and their outcomes at 6 months of age. Survivors were rare below 25 weeks (56/142, 39%), and short-term outcome of survivors was poor. Five of six (83%) born at 23 weeks had severely abnormal cranial sonograms at 6 months, as did 14 of 19 (74%) 24-week infants. In contrast, only 11 of 31 (35%) 25-week infants had grade III or IV intracranial hemorrhage or periventricular leukomalacia. These outcomes at 23 through 25 weeks' gestation are quite poor, but they are still somewhat better than those estimated by our physician sample.

So, whereas many would agree on the ability to accurately predict outcome at >=25 weeks' gestation, accurate forecasting of intact survival rates for infants born before 25 weeks is more difficult. That survivors exist in small numbers makes it difficult to accumulate large series for comparison.

Although improved survival rates are not accompanied by increased rates of major neurologic handicap, participants in this study believed that they are. Despite their pessimistic expectations, participants uncoupled these beliefs from their actions, indicating that they would administer medical management for preterm labor at very early gestational ages, including tocolysis, maturity-enhancing steroids, and transfer to a perinatal center. However, in general, they did not advoate cesarean delivery for fetal distress at gestational ages below which they believed there was likely to be a good outcome. We asked physicians to estimate the number of infants who would be expected to be free of major handicap, but there are many more less severely limited survivors [24,25]; this knowledge may have affected physicians' opinions regarding appropriate treatment of preterm labor.

There are many factors that may influence physicians to manage very-early-gestational-age deliveries in certain ways. These likely include their perceptions about outcome in terms of survival and handicap, as well

as their beliefs about the effectiveness, risks, and costs of the intervention itself. Parental desires must also influence the choices. Because no simple strategy will be optimal for all cases, it seems apparent that the most appropriate decisions will be made by parents and physicians only if they possess accurate and up-to-date information about gestational age--specific survival, long term outcome, and the effectiveness of various interventions to improve on those outcomes. We have shown that physicians performing deliveries significantly underestimate survival and handicap-free rates of premature infants. Dissemination of current neonatal outcome data may enable physicians to more accurately counsel their patients at risk for preterm delivery.

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An L-Arginine--Nitric Oxide--Cyclic Guanosine Monoph sphate System Exists in the Uterus and Inhibits Contractility During Pregnancy

[Basic Science Section]

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Outline

Graphics

Abstract

OBJECTIVE: Nitric oxide is synthesized from L-arginine and it causes relaxation of smooth muscle by elevating cyclic guanosine monophosphate levels. We hypothesized that an L-arginine--nitric oxide--cGMP system is present in the uterus and modulates contractility.

STUDY DESIGN: Isometric tension of the uterus was measured in vitro from pregnant rats in response to various agents that modulate nitric oxide--cyclic guanosine monophosphate

production or action.

RESULTS: Major findings are as follows: (1) The substrate and a donor of nitric oxide produced uterine relaxation; (2) inhibitors of the nitric oxide--cyclic guanosine monophosphate pathway blocked the relaxation responses; (3) nitric oxide synthase was localized to several uterine cell types; (4) nitric oxide was produced by the uterus during periods when L-arginine was consumed and citrulline levels increased; (5) effects of nitric oxide substrate on relaxation were mimicked by cyclic guanosine monophosphate; (6) nitric oxide--cyclic guanosine monophosphate responses were decreased during delivery; (7) L-arginine responses were increased by progesterone, and antiprogesterone treatment decreased cyclic guanosine monophosphate--induced relaxations.

CONCLUSION: An L-arginine--nitric oxide--cyclic guanosine monophosphate system is present in the uterus and it may regulate relaxation during pregnancy. The inhibitory action of L-arginine and 8-bromo-cyclic guanosine monophosphate was considerably lower during delivery and post partum, indicating that the nitric oxide system may contribute to the maintenance of uterine quiescence during pregnancy, when progesterone levels are elevated, but not during delivery. (AM J OBSTET GYNECOL 1993;170:175-85.)

Key words: Nitric oxide, uterus, contractility, pregnancy, labor

One of the most exciting recent advances in biology and medicine is the discovery that nitric oxide is produced by many cell types and that it is involved in a variety of important cellular processes, including regulation of vascular tone, platelet aggregation, neurotransmission, and immune activation [1]. Nitric oxide is an important mediator of relaxation of various smooth muscles including vascular [1] and gastrointestinal [2]. Nitric oxide is synthesized by the oxidative deamination of a guanidino nitrogen of L-arginine by at least two different isoforms of a flavin-containing enzyme, nitric oxide synthase [1]. Enzymes from brain [3] and macrophages, [4] representing constitutive and inducible forms, have recently been purified and the genes have been cloned. Synthesis of nitric oxide has been shown to be inhibited by analogs of L-arginine; N^G -nitro-L-arginine methyl ester (L-NAME) and N^G -monomethyl-L-arginine (L-NMMA). Nitric oxide is also the active component of such compounds as sodium nitroprusside and nitroglycerin [5,6]. Nitric oxide binds to heme and thus activates soluble guanylate cyclase to increase guanosine 3',5'-cyclic monophosphate (cGMP) levels [1,3,5]. Relaxation of vascular smooth muscle by nitrovasodilators is thought to be mediated by elevations in cGMP levels in the muscle cells [7,8].

Female sex steroid hormones have been shown to modulate endothelium-dependent vasorelaxation by nitric oxide; estradiol causes increased nitric oxide production whereas progesterone blunts this action, [9] suggesting that at least one form of nitric oxide synthase is regulated by these steroid hormones. Uterine contractility responses are under the dominant control of hormonal mechanisms [10]; therefore we reasoned that an L-arginine--nitric oxide system may present in the uterus and modulate uterine contractility. The current study was designed to ascertain whether an L-arginine--nitric oxide--cGMP system exists in the rat uterus and to

determine whether such a mechanism might regulate uterine contractility during pregnancy. As pharmacologic probes we used L-arginine (the substrate for nitric oxide synthesis), L-NAME (an inhibitor of nitric oxide synthase), methylene blue (a guanylate cyclase inhibitor), and 8-bromo-cGMP (a membrane-permeable stable analog of cGMP).

Material and methods

Animals. Nonpregnant (female cycling, 180 to 200 gm body weight) and pregnant Sprague-Dawley rats on day 13 to 15 of gestation (Harlan--Sprague-Dawley, Houston) were received in our animal care facility. Animals were maintained on a regimen of animal chow and water as desired. Pregnant rats were put to death on days 17, 18, 19, and 22 of gestation before labor, at the time of spontaneous delivery (one to three pups delivered) at term, or on days 1 and 2 post partum. Nonpregnant rats were ovariectomized with ketamine (50 mg/kg intraperitoneally) anesthesia (Ketalar, Parke-Davis, Morris Plains, N.J.); after 7 days of recovery they were injected subcutaneously daily for 3 days, with 1 micrograms 17beta-estradiol, 2 mg progesterone, estradiol plus progesterone in 0.2 ml of sesame oil, or vehicle only. Preterm labor and delivery in some pregnant animals were induced by intraperitoneal injections of an antiprogesterone compound (ZK 98299; onipristone, Schering AG, Berlin; 10 mg per rat in mineral oil). This treatment given on day 17 of gestation induced preterm labor and delivery on day 18. All animals were put to death in a carbon dioxide inhalation chamber.

In vitro contractility measurements. The uterus was removed and cleaned, and longitudinal strips (about 0.3 cm x 0.1 cm and 1.0 cm long) were cut with a scalpel. The strips were hung vertically in 10 ml organ baths containing Krebs solution (116 mmol/L, sodium chloride, 5.4 mmol/L potassium chloride, 2.5 mmol/L calcium chloride, 12 mmol/L monosodium acid phosphate, 11.2 mmol/L D-glucose, and 22 mmol/L sodium bicarbonate, pH 7.4, and maintained at 37 degrees C). A resting force of 0.5g was placed on each strip, and the tissues were allowed to equilibrate for 20 minutes before application of various agonists and antagonists. Force was monitored with a Grass FT 03D isometric transducer (Grass Instruments, Quincy, Mass.), connected to an eight-channel model RPS 7C Grass polygraph recorder (Grass Instruments). The contractile patterns of the strips were recorded on chart paper such that 5g force per minute was equal to a 2.5 cm^2 area. The number of minutes without contractions along the chart paper after the addition of each agent was counted. The relaxation responses were expressed as the duration (minutes) of complete inhibition of spontaneous contractility.

In some experiments, mean force developed by the muscle strip per unit time was determined by measuring the area under the contraction curves with a microcomputer-linked digitizing system (SPUC, Schering Corp., Berlin). The cross-sectional area of the tissue is estimated after the experiment from the net weight of the strip (W), its length in the bath (L),

and its density (D) by means of the following formula: Area = (W (divided by) L) (divided by) D. The density should be 1.05. The mean forces generated per unit time per cross-sectional area for each dose of the agonist are plotted to produce the dose-response curves. Spontaneous activity for each strip is recorded for 10 minutes before the addition of 8-bromo-cGMP. The force developed by the tissue strips after the 8-bromo-cGMP was added was expressed as the percentage of the force of the spontaneous activity.

Uterine tissue cultures. Full-thickness tissue (0.2 gm wet weight) from the uterus obtained from rats either on day 18 of gestation or at the time of spontaneous delivery at term was incubated in minimum essential medium (Gibco, Grand Island, N.Y.) in a carbon dioxide incubator with humidified chamber at 37 degrees C for 24 hours. After an initial 1-hour equilibration period, the medium was replaced with fresh minimum essential medium with 3 mmol/L L-arginine with or without 3 mmol/L L-NAME. After the incubations, the medium was collected for measuring nitrites, L-citrulline, and L-arginine concentrations.

Nitrite assay. Tissue culture medium nitrite concentrations were measured in triplicate by the microplate assay method and the Griess reagent. Briefly, the Griess reagent (0.5% sulfanilamide and 0.05% naphthalene diamine dihydrochloride in 2.5% orthophosphoric acid; 100 microliters) was added to 100 microliters aliquots of medium, and optical densities were measured at 550 nm in a microplate reader after a 10-minute incubation at room temperature. Nitrite values were determined with sodium nitrite used as a standard. Background nitrite values of media without tissues were subtracted from those with tissues, and values were expressed as nanomoles per gram wet weight of the uterine tissue.

L-Citrulline and L-arginine determinations. The tissue culture medium (50 microliters) was mixed (1:1, vol/vol) with 3.75% sulfosalicylic acid in lithium buffer (Beckman, Palo Alto, Calif.) containing 4.0 nmol 4-pyridylethyl-L-cysteine as an internal standard and mixed thoroughly to precipitate proteins. After centrifugation of the pellet, the supernatant was analyzed in an amino acid analyzer (model 6300, Beckman) for L-citrulline and L-arginine concentrations.

Nicotinamide dinucleotide phosphate (NADPH) diaphorase staining. The uterus was opened longitudinally along the mesenteric border. After the fetuses, placenta, and membranes were removed, the resultant sheet of uterus was stretched and pinned out on a wax sheet. These whole mounts were immediately fixed in 4% (wt/vol) paraformaldehyde in phosphate-buffered saline solution, pH 7.4, for 20 minutes at room temperature. After washing in phosphate-buffered saline solution two times, whole-mount tissues were stained for NADPH diaphorase activity by incubation in 50 mmol/L Tris-hydrochloride, pH 8, containing 1.0 mmol/L beta-NADPH, 0.5 mmol/L nitroblue tetrazolium, and 0.25% Triton X-100 for 30 minutes at 37 degrees C. Tissues were washed in phosphate-buffered saline solution and examined directly with light microscopy. The reaction

product of NADPH diaphorase appeared as a dark blue deposit. Tissues were exposed to the staining solution without NADPH, as controls for the staining procedure.

Chemicals. All the chemicals were analytic grade, and the drugs that were used in these studies were purchased from Sigma Chemical Co. (St. Louis). The agents used were: L-arginine, L-NAME, sodium nitroprusside, methylene blue, and 8-bromo-cGMP.

Statistics. Data are expressed as means +-\SEM. Levels of significance were determined by the analysis of variance and Duncan's multiple range test or the Student t test.

Results

Effects of L-arginine and L-NAME on uterine contractility. Application of 3 mmol/L L-arginine to muscle strips caused immediate relaxation of the spontaneous contractile activity of uterine tissue strips obtained from 18-day-pregnant rats (Fig. 1), and these relaxation responses were repeatedly inducible in each strip without washout. These results indicate that an L-arginine--nitric oxide system may exist in the uterus and induce relaxation of uterine muscle activity. The specificity of the nitric oxide--generating system was examined by using a compound that is known to inhibit the nitric oxide synthetic pathway. L-NAME, the structural analog of L-arginine and a competitive inhibitor of nitric oxide synthesis when added to the muscle bath during the relaxation phase, produced a rapid reversal of the effects of L-arginine (Fig. 1, B) confirming the existence of an L-arginine--nitric oxide system in the pregnant rat uterus. In addition, L-NAME alone caused an increase in the contractile activity of the pregnant rat uterus. The effects of L-arginine and L-NAME presented in Fig. 1 are representative contractile tracings from 12 uterine strips of 6 pregnant rats on day 18 of gestation. In 6 strips from 3 animals, the endometrium was gently stripped away from the myometrium. The addition of L-arginine to the isolated myometrial strips produced relaxation comparable to that observed in whole, intact uterine tissues, as described. Also, in very small strips (n = 11; about 0.08 x 0.04 x 0.5 mm) of isolated myometrium, with a negligible amount of vascular tissue, L-arginine produced relaxation.

Sodium nitroprusside effects on uterine contractility. To further investigate the ability of nitric oxide to induce relaxation of the uterine tissue, we examined the effects of a nitric oxide donor, sodium nitroprusside. When sodium nitroprusside (dose range 0.1 to 5.0 mmol/L) was applied to the uterine strips from 18-day-pregnant rats, after a short delay, the spontaneous contractile activity was completely abolished for prolonged periods of time, but these tissues were still responsive to potassium chloride (Fig. 2, A and B). Fig. 2 represents a typical response to sodium nitroprusside (5 mmol/L) in 12 strips from 4 animals. The results with potassium chloride show that the muscle was not damaged by sodium nitroprusside treatment.

Effects of methylene blue on L-arginine--induced uterine relaxation. Nitrovasodilators and nitric oxide have been shown to activate guanylate cyclase and enhance the production of cGMP, and an increase in cGMP could result in inhibition of uterine myometrial contractility. We used methylene blue, which inhibits guanylate cyclase, to determine whether this compound could mimic the effects of inhibitors of the nitric oxide synthetic pathway in the uterus. Uterine strips were exposed to methylene blue for 15 to 20 minutes before the addition of 1 mmol/L L-arginine. The duration of inhibition of contractility, after the addition of L-arginine, was 5.8 +-\0.3 minutes, which was significantly ($p < 0.001$) inhibited by 0.1 mmol/L methylene blue (0.4 +-\0.1 minutes). Methylene blue at 0.01 mmol/L was ineffective (6.1 +-\0.3 minutes).

Dose-dependent effects of L-arginine on uterine contractility during pregnancy, during spontaneous labor, and post partum. In these experiments we examined the possibility that the relaxation responses of L-arginine might be reduced during labor. The effects of varying doses of L-arginine (0.1 mmol/L to 10 mmol/L) on spontaneous uterine contractility were measured in uterine strips from pregnant rats obtained on days 17, 18, and 19, and on day 22, either before or during spontaneous delivery (1 to 3 pups delivered), and on days 1 and 2 post partum. Fig. 3 shows dose-dependent effects of L-arginine on the duration of inhibition of contractile activity. L-Arginine evoked relaxation of spontaneous uterine contractility in a concentration-dependent manner. The duration of inhibition of the spontaneous contractile activity at each of the effective concentrations of L-arginine (0.6 mmol/L to 10 mmol/L) was significantly (analysis of variance, $p < 0.05$) shorter in rats delivering spontaneously at term (day 22) than in rats not in labor at term (day 22) or before term (days 17, 18, and 19 of gestation). Thus tissues from nondelivering rats both on day 22 and on days 17 to 19 of pregnancy were more responsive to L-arginine--induced uterine relaxation. Effects of L-arginine on the duration of inhibition of the spontaneous contractility of the uterus were further decreased in rats on postpartum days 1 and 2.

Dose-dependent effects of L-arginine on uterine contractility in steroid hormone--treated nonpregnant rats. To determine whether steroid hormones might regulate the L-arginine--nitric oxide responses, we examined the effects of estradiol and progesterone on the L-arginine--induced relaxation responses of the nonpregnant uterus. Adult female rats were ovariectomized and injected daily subcutaneously with 17beta estradiol (1 micrograms per rat), progesterone (2 mg per rat), estradiol plus progesterone in sesame oil, or oil alone for 3 days. Effects of varying doses (0.1 mmol/L to 10 mmol/L) of L-arginine on the spontaneous contractile activity of the uterus were determined. L-Arginine effects measured as the duration of complete inhibition of spontaneous contractions were dose dependent. The duration (in minutes) of inhibition of contractility at each L-arginine concentration used was similar in ovariectomized, nonpregnant rats receiving estradiol, estradiol plus progesterone, or oil only. However, the relaxation responsiveness to L-arginine at the 10 mmol/L dose was greater in

progesterone-treated rats (22.5 +-\6.0 minutes), and this was significant (analysis of variance, $p < 0.05$) when compared with that of estrogen-treated animals (6.1 +-\2.0 minutes) but not compared with that of control (10.5 +-\4.1 minutes) or estrogen plus progesterone--treated (10.0 +-\5.1 minutes ($p < 0.15$) animals.

Dose-dependent effects of 8-bromo-cGMP on uterine contractility during pregnancy, spontaneous labor, and preterm labor. To examine whether cGMP relaxation responses were changed during term and preterm delivery, we measured contractile activity to a stable, membrane-permeable cGMP analog. 8-Bromo-cGMP log dose contractile-response curves for uterine tissues from animals delivering either spontaneously at term or after preterm induction with antiprogesterone on day 18 of gestation and from control animals on day 18 of gestation are shown in Fig. 4. Tissues from control animals relaxed with 8-bromo-cGMP at a median effective dose (mean +-\SEM) of 2.1 +-\0.5 x 10^{-6} mol/L. However, the tissue from animals delivering at term or preterm after onipristone was significantly ($p < 0.001$) less reactive to 8-bromo-cGMP, with median effective dose values of 0.91 +-\0.1 x 10^{-4} and 0.46 +-\0.08 x 10^{-4} mol/L, respectively. These results indicate that the tissue relaxation responsiveness to 8-bromo-cGMP is substantially reduced during term and preterm labor.

Nitrite production from uterine tissue obtained from pregnant and spontaneously delivering rats. To determine whether nitric oxide is produced by the uterus, we measured nitrite formation by uterine tissues in 24-hour organ cultures. Nitrite is an oxidation product of the L-arginine--dependent nitric oxide synthase pathway, and nitrites are detected spectrophotometrically with the Greiss reagent method. Data presented in Fig. 5 show that nitrites are produced by the rat uterus in the presence of L-arginine, and this production is substantially ($p < 0.01$) inhibited by L-NAME. Nitrite production by the uterus was not significantly different ($p = 0.08$) during term delivery compared with that during preterm delivery.

L-Citrulline and L-arginine concentrations in cultures of uterine tissues from pregnant and delivering rats. L-Citrulline and L-arginine concentrations in the tissue culture medium were measured to estimate the nitric oxide--producing activity of the uterus. Citrulline is relatively stable metabolically and can be used for measuring nitric oxide synthetic activity. L-Citrulline was secreted by the uterine tissues in the presence of L-arginine, and this secretion was completely abolished in the presence of L-NAME (Fig. 6, A), suggesting that L-citrulline in the media is synthesized through a nitric oxide synthetic pathway from L-arginine. In the absence of L-NAME, L-arginine concentrations decreased with a corresponding increase in L-citrulline concentrations, indicating the utilization of L-arginine by this tissue (Fig. 6, B). However, L-NAME abolished L-arginine utilization and L-citrulline formation. Although there appears to be a decrease in the production of citrulline by the ut rus from delivering rats, it is not statistically significant ($p = 0.07$). To ascertain whether

L-arginine was rapidly utilized by the uterus in muscle baths, we measured L-arginine in bath solutions. There were no changes in L-arginine concentrations at 1, 2, 5, 10, 30, and 60 minutes after the addition of L-arginine at a given dose. During the same periods, citrulline concentrations were undetectable in the baths.

NADPH diaphorase staining. We examined whole-mount preparations of uterine tissues from rats on day 18 of gestation and during spontaneous delivery for the presence of nitric oxide synthase activity. NADPH diaphorase staining was present in the myometrium, endometrium, blood vessels, and nerves (Fig. 7), indicating the presence of nitric oxide synthase activity in all compartments of the uterus. Intense staining was present in muscle bundles in addition to uterine epithelial and vascular endothelial cells. The staining of the cervical ganglion obtained from the same animal, shown in Fig. 7, indicates the high density of the diaphorase staining in cervical nerves. Staining was absent in tissues exposed to the staining solution without NADPH.

Comment

In the current study we demonstrated that an L-arginine--nitric oxide--cGMP system is present in the uterus and that it modulates uterine contractility. The major findings from this study are as follows: (1) that the substrate (L-arginine, Fig. 1) and a donor (sodium nitroprusside, Fig. 2) of nitric oxide produce substantial relaxation of the pregnant rat uterus, (2) that inhibitors of nitric oxide synthase (L-NAME, Fig. 1) and soluble guanylate cyclase (methylene blue) block the effects of L-arginine, (3) that nitric oxide synthase activity is localized to most cell compartments of the uterus (Fig. 7), (4) that nitric oxide is produced by uterine tissue in culture (Figs. 5 and 6), (5) that the effects of nitric oxide substrate are mimicked by cGMP (Fig. 4), (6) that nitric oxide--cGMP responses are decreased (Figs. 3 and 4) at the time of delivery and post partum, and (7) that nitric oxide--cGMP responses are increased by progesterone and decreased by antiprogesterone (Fig. 4). These studies provide strong evidence of the existence of a nitric oxide--cGMP system in the uterus and indicate that responsiveness to this system declines during labor.

L-Arginine has been extensively used to study nitric oxide and its effects on smooth muscle contractility in other tissues. In this study addition of L-arginine to uterine strips caused substantial relaxation of the tissue, similar to that described for smooth muscle of vascular, [1] gastrointestinal, [2] tracheal, [11] and cavernous [12] tissues. Several studies [1,6,8] have demonstrated that L-arginine produced relaxation of vascular smooth muscle through the generation of nitric oxide and that this effect was blocked by the inhibitors of nitric oxide synthesis [13,14]. L-NAME, L-NMMA, and other L-arginine analogs inhibit nitric oxide production and relaxation in the vascular tissue through inhibiting nitric oxide synthase. These analogs also inhibit nitric oxide synthase in other cells,including brain macrophages. The ability of L-arginine to produce rapid relaxation in

the uterus, together with the observation that L-NAME reversed the effects of L-arginine, provides convincing evidence for the generation of nitric oxide in the L-arginine--induced relaxation response of the rat uterus. In addition, after a short period L-NAME alone slightly increased the contractile activity of the uterus (Fig. 1, C) indicating that inhibition of nitric oxide synthase may be responsible for this increased activity. Similar increases in contractile activity were reported for other smooth muscle cells [2]. However, this increase occurs in the absence of exogenous L-arginine. Therefore endogenous substrate must be contributing to the generation of nitric oxide. This phenomenon needs to be examined further to fully clarify the mechanism.

Nitric oxide is synthesized by the oxidative deamination of a guanidine nitrogen of L-arginine by nitric oxide synthase [1]. This enzyme has been reported to exist in several isoforms, and at least two isoforms, constitutive and inducible, have been isolated [3,4] and cloned [15,16]. The constitutive isoform is Ca^{++} and/or calmodulin dependent and is expressed constitutively in endothelial and brain tissues [1]. The inducible isoform of nitric oxide synthase is Ca^{++} independent, is induced by various cytokines and bacterial lipopolysaccharide, and is present in many cells, including macrophages, endothelial cells, and liver cells [1]. The rapid induction of relaxation of L-arginine indicates that the nitric oxide synthase in the uterus is a constitutive form. However, further studies are required to characterize the isoforms of the enzyme in this tissue.

NADPH diaphorase staining has been extensively used to localize nitric oxide synthase activity in a variety of tissues [17,18]. Using this method we demonstrated the presence of nitric oxide synthetic activity localized to myometrium, endometrium, blood vessels, and nerves in the uterus, indicating that these tissues possess the nitric oxide synthase necessary for production of nitric oxide. In immunohistochemical studies nitric oxide synthase and NADPH diaphorase staining have previously been shown to be colocalized in neurons [17] and in human kidney cells transfected with nitric oxide synthase, [18] suggesting that nitric oxide activity was fully responsible for the NADPH diaphorase reaction. The major cellular mass in the uterine strips used in our studies for measuring contractility is circular and longitudinal muscle cells, and therefore it is possible that nitric oxide is produced by L-arginine in muscle cells, as well as in other cells.

Further evidence for the presence of a nitric oxide system in the uterus is demonstrated by the nitrite production by this tissue. Nitric oxide is generated from L-arginine and is rapidly converted to stable products, nitrites, and nitrates. Nitrite accumulation in the culture medium has been used as a measure of nitric oxide production in other tissues [19]. Nitrites were produced by the uterus in the presence of L-arginine and they were inhibited by L-NAME, indicating the involvement of nitric oxide synthase in the generation of nitric oxide. Both delivery and nondelivery tissues produced similar amounts of nitrites, indicating that nitric oxide synthase activity is not different in these tissues. Therefore the decrease in the

responsiveness to L-arginine during delivery does not appear to be due to a decrease in the generation of nitric oxide.

Additional support for the presence of nitric oxide synthase activity in the rat uterus is provided by the production of citrulline and the utilization of L-arginine. Citrulline production has been used as a measure of nitric oxide production from L-arginine [20]. Citrulline was increased in the presence of L-arginine, with a corresponding decrease in L-arginine in the culture medium. L-NAME reversed these changes in concentrations, indicating the presence of nitric oxide synthetic pathway. Furthermore, measurement of citrulline and L-arginine concentrations in muscle baths during recording of contractility showed that there was no decrease in L-arginine after a given dose, up to a period of 1 hour. The observation that a second cumulative dose of L-arginine produced a second relaxation in the absence of a decrease in L-arginine in the muscle baths indicates that perhaps nitric oxide synthase may be desensitized in vitro after a short period and reset to a higher threshold level for the substrate.

Nitric oxide donors are thought to cause relaxation of smooth muscle tissue by liberating nitric oxide. In the current study sodium nitroprusside produced substantial relaxation of the rat uterus, indicating that uterine tissue is responsive to the nitric oxide liberated from an exogenously added nitric oxide donor. These observations are consistent with sodium nitroprusside effects on gastrointestinal and vascular smooth muscle tissue [1,2]. However, our results are somewhat at variance with one study that reported a lack of effect of similar doses of sodium nitroprusside on the rat uterus [21]. However, the physiologic state of the animals was not clearly stated in this study. Our study shows that sodium nitroprusside causes relaxation of the uterus in pregnant animals, but it might not do so in nonpregnant ones. There was a consistent lag period before the onset of relaxation by sodium nitroprusside, and this was considerably shorter (21.8 +-\3.4 minutes) in 18-day-pregnant rats than during delivery (99.0 +-\18 minutes, $p < 0.001$), further supporting the concept that the relaxation by sodium nitroprusside may depend on the physiologic state of the animal. The facts that sodium nitroprusside was ineffective at low doses (<1 mmol/L) and required a relatively high concentration (5 mmol/L) to produce relaxation and that it has a long lag period to onset indicate that it may not be an efficient nitric oxide donor in the uterus. A possible explanation for the long lag period to onset of relaxation and the need for high concentrations is the way nitric oxide is liberated from sodium nitroprusside. The mechanism(s) by which sodium nitroprusside is converted to nitric oxide has not been defined. Previously, it was assumed that conversion occurs by spontaneous chemical degradation of sodium nitroprusside to nitric oxide [22]. However, recently it has been suggested that sodium nitroprusside is stable in solution and that nitric oxide release is undetectable [23]. It has been shown that nitric oxide generation from sodium nitroprusside in arterial smooth muscle cells requires metabolic activation, and the site of conversion was thought to be membrane associated [24]. A similar metabolic activator mechanism may also be

required in the uterus. The fact that vascular tissue reacts to sodium nitroprusside at low doses (<1 micromole/L) compared with those for uterine tissue indicates that this conversion system may be more active in vascular tissues.

It is now well recognized that nitric oxide binds to heme and activates soluble guanylate cyclase [1,3] thereby causing a rise in cGMP levels [7,8]. In most smooth muscle cells this process is thought to lead to relaxation [6,7]. There is also much evidence that stimulation of guanylate cyclase activity by nitric oxide and the consequent smooth muscle relaxation can be blocked by methylene blue [1,25]. The relaxation of the uterus by L-arginine was antagonized by methylene blue in the current investigation, indicating that nitric oxide responses of the myometrtium are also mediated through cGMP.

Our observations also provide indirect evidence for a role for cGMP in mediating relaxation effect of nitric oxide in the uterus, consistent with those in other tissues [1,2]. A role for cGMP in uterine relaxation is supported by several lines of evidence [26,27]: (1) Nitrovasodilators such as nitroglycerin, sodium nitroprusside, and hydroxylamine increase cGMP levels in rat uterus; (2) 8-bromo cGMP can relax rat myometrial strips. However, the dose-response relationship between the ability of the guanylate cyclase activators to elevate cGMP levels and their ability to relax the uterus have been questioned, [26] primarily on the basis of data showing that sodium nitroprusside was unable to induce immediate relaxation despite an increase in cGMP levels. However, as noted here, the effects of sodium nitroprusside may depend on the physiologic state and the presence of the metabolic activator system. The ability of cGMP to relax uterine muscle may also relay on similar conditions and be more responsive during pregnancy. In fact, our studies indicate that relaxation responses of the uterus are highly sensitive to cGMP during pregnancy. However, there is a tremendous decrease in sensitivity to cGMP at the time of delivery (both spontaneous and preterm). This suggests that there are changes in the cGMP effector system during term and preterm labor.

The L-arginine effects on uterine relaxation were dose dependent in concentrations from 0.1 mmol/L to 10 mmol/L. The minimum dose of L-arginine (0.1 mmol/L) that produced significant relaxation responses in our studies is somewhat higher than those reported for other smooth muscle tissues (0.1 micromole/L to 100 micromole/L), [2,6] but these concentrations are within the physiologic range (0.2 to 3.0 mmol/L) reported for levels of L-arginine in rat serum [28]. The duration of inhibition of uterine contractility was progressively shorter in delivering (day 22) and postpartum rats compared with that in rats that were not in labor either at term on day 22 or before term on days 17 to 19 of gestation. Large differences between day 22 nondelivering animals versus day 22 delivering animals indicate that the change in L-arginine responsiveness occurs within the last few hours of pregnancy. These L-arginine effects were diminished further by 1 day post partum and were almost completely absent by the second day post partum. These studies indicate that an L-arginine--nitric

oxide--cGMP system may contribute to the quiescent state of uterus during pregnancy. Conversely, it is possible that the nitric oxide synthetic pathway and the relaxation response may be subdued in uterine tissue during labor and pave the way for an increased contractile activity. On the basis of the study, we propose that locally produced nitric oxide in the pregnant uterus contributes to the quiescent state of this tissue during the gestational period and that diminution in the responsiveness of the uterus to nitric oxide at term could lead to increased uterine contractility and the initiation of labor. Thus the nitric oxide systems appear to be a key regulator of uterine contractions. In addition, nitric oxide may also have other roles, including an immunologic role, during pregnancy.

The steroid hormones estradiol and progesterone play a significant role in the maintenance of pregnancy and initiation of labor [10]. In this study L-arginine--nitric oxide effects on uterine relaxation were substantially increased by progesterone in nonpregnant animals but not by estradiol or by estradiol plus progesterone. Therefore, if one extrapolates these data to pregnancy, nitric oxide may be responsible for uterine quiescence during the preterm period when progesterone levels are elevated. A decrease in progesterone concentration at term and post partum may contribute to the decreases in the relaxation responses to L-arginine--nitric oxide and cGMP. It is not evident how progesterone may modulate this response, but it could control nitric oxide synthase as suggested in vascular tissues [9] or it might regulate cGMP availability or effects. Moreover, our studies suggest that progesterone alone may not be sufficient to fully elicit L-arginine responses in nonpregnant animals, because these responses were significantly lower than those in pregnant animals. Our study provides evidence that cGMP effects on the relaxation of the uterus were reduced by an antiprogestin and at term during periods of low circulating levels of progesterone, indicating that progesterone may modulate the uterine responsiveness to nitric oxide by regulating the cGMP effector system.

In summary, our results suggests that an L-arginine--nitric oxide--cGMP system exists in the pregnant rat uterus and that it causes relaxation of the uterus during pregnancy. The L-arginine--nitric oxide--induced relaxation responses are reversed by the inhibitors of both nitric oxide synthesis and guanylate cyclase, and nitric oxide is produced by the uterus, thereby providing more evidence for this pathway in the uterus. Further, the relaxation effects of L-arginine--nitric oxide and cGMP are substantially decreased during both term and preterm delivery and post partum, indicating that nitric oxide--cGMP may contribute to the maintenance of uterine quiescence during pregnancy, when progesterone levels are elevated, but not during delivery.

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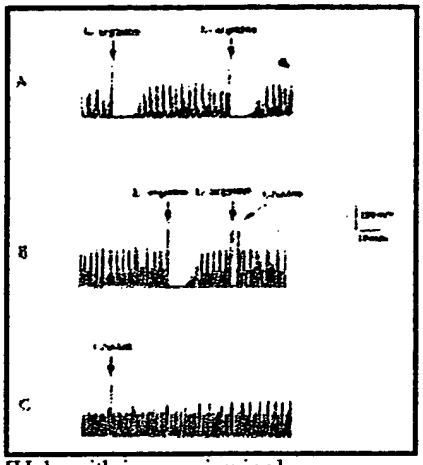

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Figure 1. Effect of L-arginine and L-NAME on spontaneously contracting uterine strips from rat uterus obtained on day 18 of pregnancy. Application of L-arginine (3 mmol/L) to muscle bath caused immediate relaxation (10 to 15 minutes' duration) of contractility (A). Effect of L-arginine (3 mmol/L) was antagonized by L-NAME (3 mmol/L) when added during an L-arginine–induced relaxation (B). Applications of L-NAME (0.1 mmol/L) to muscle bath increase contractile activity (C). These are typical recordings, and each upstroke from baseline represents a contraction. Similar responses were observed in 12 uterine strips from 6 animals

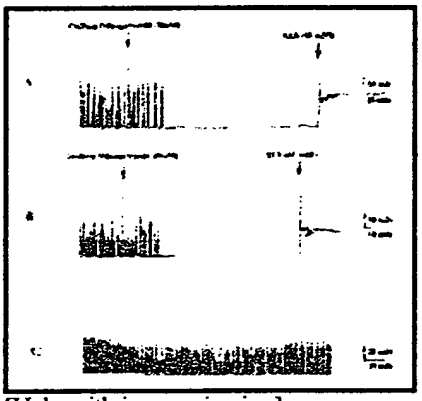

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Figure 2. Sodium nitroprusside, a nitric oxide donor, caused relaxation of spontaneously contracting pregnant rat uterus on day 18 of gestation. Application of sodium nitroprusside caused sustained relaxation in spontaneously contracting uterine strips after a lag period (A and B). Tissues in relaxed state after nitroprusside were responsive to potassium chloride (KCl). C, Temporal control strip not receiving sodium nitroprusside. Similar recordings of 12 uterine strips from 4 animals were obtained

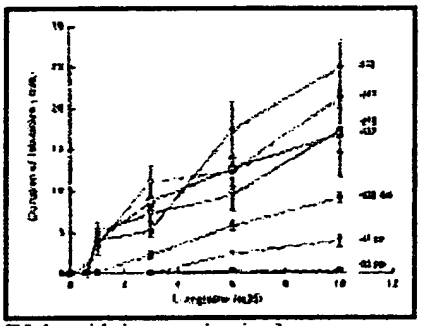

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Figure 3. Dose-dependent relaxation effects of L-arginine (0.1 mmol/L to 10 mmol/L) on spontaneously contracting uterine strips from rats at different stages of gestation, during delivery, and post partum. Tissues were obtained on days 17 to 22 of gestation (d17, d18, d19, and d22), on day 22 during spontaneous delivery (d22 del, 1 to 3 pups delivered), or at 1 (d1 pp) and 2 (d2 pp) days post partum. Duration of complete inhibition of spontaneous uterine contractions is dose dependent. Data were analyzed by repeated measures analysis of variance on 7 groups. Effects of L-arginine from concentrations of 1 mmol/L are significantly ($p < 0.01$) decreased during spontaneous delivery at term and post partum, compared with all other times. Each data point represents mean +-\SEM. Total number of strips studied at each time period was 8 to 16 from 4 to 6 animals per group

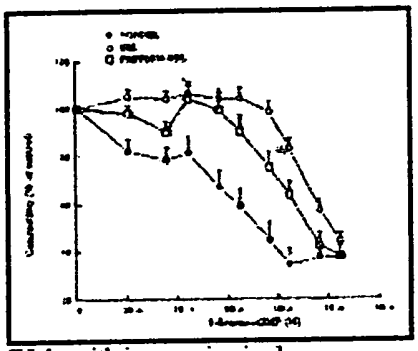

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Figure 4. 8-Bromo-cGMP dose relaxation-response curves for uterine tissues from rats delivering spontaneously at term (DEL), delivering preterm with onipristone (PRETERM DEL), and not delivering on day 18 of gestation (NONDEL). Each point represents mean +-\SEM for 4 strips from each animal for 4 rats per group

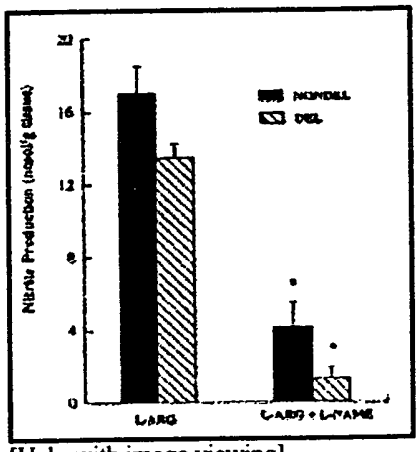

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Figure 5. Nitric oxide–generating activity measured as nitrite production in uterus from nondelivering animals on day 18 of gestation (NONDEL) and spontaneously delivering rats (DEL) Uterine tissue was incubated with 3 mmol/L L-arginine (L-ARG) with and without 30 mmol/L L-NAME, and nitrite production during 24-hour period is presented as mean +-\SEM for tissues from 8 animals in each group. Asterisk, Significantly different from respective groups with L-arginine only ($p < 0.01$)

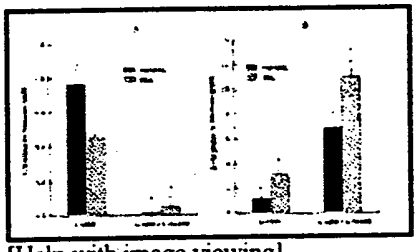

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Figure 6. L-Citrulline (A) and L-arginine (B) concentrations (millimoles per liter per milligram of tissue) in tissue culture media incubated with uterine tissue from nondelivering rats on day 18 of gestation (NONDEL) and spontaneously delivering rats (DEL). Treatments were same as in Fig. 5. Asterisk, Significantly different from respective groups with L-arginine only ($p < 0.01$)

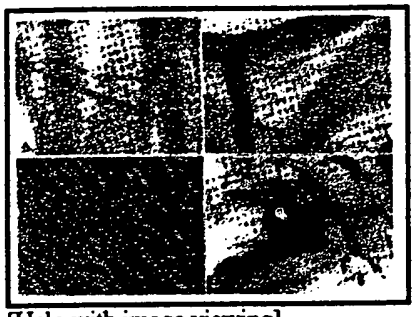

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Figure 7. NADPH diaphorase histochemical staining of nitric oxide synthase activity in uterus and cervical ganglion from pregnant (day 18) rat. Whole-mount preparation of uterus was fixed for 30 minutes in 4% paraformaldehyde, and tetrazolium blue was reduced in presence of NADPH. Intense staining of myometrium, small blood vessels, and cervical nerves (A and B), endometrium (C), and cervical ganglion (D) is demonstrated. A, Isolated myometrial tissue showing bundles of smoooth muscle cells (SM, vertical bands), blood vessels (B), and nerves (N). B, Large blood vessel (top left branching to bottom left) showing staining of endothelium (interior). C, Staining of epithelial cells in endometrium. D, Intense staining of ganglia of cervical ganglion. (Original magnification: A, C, and D, x 160; B, x 380.)

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Inhibition of Nitric Oxide Synthesis in Rats During Pregnancy Produces Signs Similar to Those of Preeclampsia

[Basic Science Section]

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Outline

Graphics

Abstract

OBJECTIVES: Preeclampsia is associated with hypertension, fetal growth retardation, and proteinurea. We hypothesized that impaired vascular nitric oxide synthesis during pregnancy may be an important causal factor in preeclampsia.

STUDY DESIGN: An inhibitor of nitric oxide synthase, L-nitro-arginine methyl ester, or a nitric oxide donor, nitroglycerin, was infused subcutaneously to rats at a constant rate from day 17 of gestation. Systolic blood pressure, day of spontaneous delivery, weight, and mortality rate of pups were recorded.

RESULTS: Systolic blood pressures in rats infused with L-nitro-arginine methyl ester at

daily doses of both 25 and 50 mg were significantly elevated compared with controls. This treatment also caused a substantial decrease in the weight of pups, with an increase in mortality rate, without affecting the gestational length. These effects were dose dependent. Nitroglycerin infusion, on the other hand, affected neither the weight and mortality rate of the pups nor the length of gestation.

CONCLUSIONS: Infusion of an inhibitor of nitric oxide synthesis during pregnancy causes hypertension and fetal growth retardation, without affecting gestational length. These signs are similar to those of preeclampsia and indicate that an alteration in nitric oxide synthesis may be one of the factors responsible for this disorder. Treatment with nitric oxide inhibitors may be used in an animal model for preeclampsia, to test various therapeutic strategies. (AM J OBSTET GYNECOL 1993;169:1316-20.)

Key words: Nitric oxide, pregnancy, preeclampsia, fetal growth

Preeclampsia, synonymous with toxemia or pregnancy-induced hypertension, is considered one of the most significant health problems in human pregnancy. This condition is generally recognized in the latter half of gestation and is reported to affect between 7% and 30% of all women [1]. It is the leading cause of fetal growth retardation and infant morbidity and mortality associated with premature delivery and maternal death [2]. It is estimated that 20% to 25% of total perinatal mortality is caused by pregnancy-associated hypertensive disorders [3]. Hypertension, decreased fetal growth, and proteinuria are the hallmarks of preeclampsia [3]. Although the precise causal factors are unknown, analysis of the pathophysiologic mechanisms of this disorder has focused on elevated blood pressure associated with activated hemostatic system, endothelial cell injury, altered prostacyclin/thromboxane ratios, and abnormal placental morphologic conditions.

During normal pregnancy several hemodynamic changes occur, including profound increases in blood flow through uterine blood vessels, altered responses to vasopressor agents, and reduced peripheral resistance and blood pressure [4]. The mechanism(s) for these changes are unknown. Increased synthesis of prostacyclin (PGI_2, a vasodilator) with a resultant dominance of the vasodilatory effects of PGI_2 over thromboxane A_2 (TxA_2, a vasoconstrictor) has been proposed as a possible mechanism for vascular changes during pregnancy and for reduced sensitivity of the maternal vascular system to angiotensin II [5,6,7]. On the other hand, an imbalance in the ratio of TxA_2 to PGI_2 in favor of the vasoconstrictor action of TxA_2 has been proposed as a mechanism for the major clinical symptoms of preeclampsia [8]. Although a disturbance in the levels of PGI_2 and TxA_2 suggests an explanation for elevated blood pressure during pregnancy, various other factors have also been proposed [9].

Nitric oxide synthesized from L-arginine acts through the stimulation of the soluble guanylate cyclase to increase cyclic guanosine 3',5'-monophosphate levels in vascular smooth muscle to produce relaxation [10,11].

Administration of N^G -nitro-L-arginine methyl ester, a potent inhibitor of nitric oxide causes a long-lasting blood pressure increase [12] and suggests that a reduction in the synthesis of nitric oxide may contribute to the pathogenesis of hypertension [13]. Furthermore, it potentiated pressor responses to angiotensin II, vasopressin, and norepinephrine [14]. In patients with pregnancy-induced hypertension release of nitric oxide by umbilical vessels is blunted, [9] and the physiologic decrease in blood pressure in pregnant spontaneous hypertensive rats was shown to depend on endothelial nitric oxide release [15]. These studies support the hypothesis that impaired nitric oxide synthesis may be an important factor in the cause of pregnancy-induced hypertension. However, other studies suggest that manipulations of nitric oxide do not produce alterations in umbilical vessel contractions in vitro [16,17].

Thus the role of nitric oxide in pregnancy-induced hypertension is unclear, and, aside from a study by Molnar and Hertelendy, [14] there have been no studies of the effects of nitric oxide inhibitors during pregnancy. The objective of this was to determine the effects of a constant infusion of an inhibitor of nitric oxide production during late pregnancy on the blood pressure, fetal development, and pregnancy outcome. Our study shows that inhibition of nitric oxide during pregnancy (1) produces signs similar to those of preeclampsia, (2) may be an effective model for further animal studies of preeclampsia, and (3) fails to alter the normal timing of parturition.

Material and methods

Adult virgin pregnant rats (300 to 325 gm body weight) were purchased from Harlan-Sprague-Dawley (Houston) and were received in our animal care and use facility on day 16 of pregnancy (day 1 = day of positive sperm smear). All animals were given free access to food and water. On day 17 of pregnancy systolic blood pressure was measured with a pneumatic tail-cuff device (Narco-BioSystems, Houston) in animals that had been placed on a heating blanket and prewarmed in a metal chamber maintained at approximately 30 degrees C. Blood pressure values obtained from three consecutive measurements were averaged and recorded as the pressure of a given rat at each time point. Starting on day 17 of pregnancy, 8 to 10 rats per group received infusions by means of osmotic minipumps of the specific inhibitor of nitric oxide synthase, N^G -nitro-L-arginine methyl ester (Sigma, St. Louis), at 25 or 50 mg/day per rat dissolved in sterile saline solution or nitroglycerin (ICI Americas, Wilmington, Del.), a nitric oxide donor, at 240 or 480 micrograms/day per rat. Osmotic minipumps (Alza, Pala Alto, Calif., No. 2ML2, 5 microliters/hr) were filled with vehicle with or without L-nitro-arginine methyl ester or nitroglycerin and placed subcutaneously during ketamine (Ketalar, Parke-Davis) anesthesia. Measurement of systolic blood pressure of all animals receiving L-nitro-arginine methyl ester was also carried out on days 18 and 22 of pregnancy. Animals were monitored every hour during the day throughout the treatment preriod. After delivery of the pups (within 1 hour), the number, weight, and condition of the pups

were recorded.

Aliquots of urine were collected from five rats each in the control group and the group treated with 50 mg of L-nitro-arginine methyl ester. Animals were lightly anesthetized with halothane (Halocarbon, North Augusta, S.C.), and a flexible polyethylene tubing (0.965 mm outer diameter, 0.58 mm inner diameter, Becton Dickinson, Parsippany, N.J.) was passed through the urethra into the bladder and drops of urine were collected. Protein concentrations of the aliquots of urine were analyzed by BioRad protein assay kit (BioRad, Richmond, Calif.). This kit, which is based on the Bradford method, has been used previously for urine protein determinations [18]. All procedures were approved by the Animal Care and Use Committee of The University of Texas Medical Branch.

The mean (+-\SEM) values for the day of pregnancy when parturition occurred, the number of pups delivered, and weight of pups were calculated. Statistical analysis was performed with analysis of variance, with multiple comparisons of means where appropriate. Mortality rates as the proportion of live/dead pups were analyzed with the chi squared (chi^2) test.

Results

Systolic blood pressure measured in animals infused with L-nitro-arginine methyl ester or saline solution on days 17, 18, and 22 of pregnancy is presented in Fig. 1. Basal systolic blood pressure was similar in all pregnant animals on day 17 of pregnancy, before treatment (overall mean +-\SEM = 115.6 +-\2.1 mm Hg). Infusion of L-nitro-arginine methyl ester significantly, and in a dose-dependent manner, increased the blood pressure by day 18 of pregnancy (Fig. 1). Blood pressure in animals receiving it at 25 mg/day (144 +-\4) and 50 mg/day (160 +-\2) was significantly ($p < 0.001$) higher compared with that of rats receiving saline solution only (116.3 +-\2.0). Blood pressure remained elevated throughout the treatment period in animals receiving 25 or 50 mg per day, as evidenced by the blood pressure measurements on day 22 of pregnancy (Fig. 1).

All the animals delivered spontaneously at term. The mean (+-\SEM) day of pregnancy when parturition occurred in vehicle-infused animals was day 22.3 +-\0.2, which was not significantly different in animals infused with either L-nitro-arginine methyl ester or nitroglycerin Table I. However, the weight of the pups of the L-nitro-arginine methyl ester --treated animals was different. L-nitro-arginine methyl ester infusion, in a dose-dependent manner, caused a significant ($p < 0.01$) reduction in the weight and size of the pups Table Iand Fig. 2). Animals receiving 25 mg L-nitro-arginine methyl ester per day had pups with a mean (+-\SEM) weight (grams) of 5.1 +-\0.1 that was significantly ($p < 0.001$) lower than that of those receiving saline solution (6.2 +-\0.1). Rats receiving 50 mg L-nitro-arginine methyl ester per day had pups (4.6 +-\0.1) that were significantly ($p < 0.001$) smaller than those receiving either 25 mg of L-nitro-arginine methyl ester per day or vehicle. Animals that received either 240 or 480 micrograms of

nitroglycerin delivered pups that were not significantly different in weight from the vehicle-infused rats Table I. Animals in all the groups delivered a similar number of pups Table I.

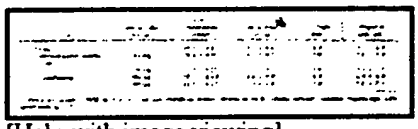

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Table I. Effects of L-nitro-arginine methyl ester or nitroglycerin infusions to pregnant rats on day of delivery and number, weight, and mortality of pups

The mortality of the pups was examined immediately after parturition. A significant number of pups were born dead in the L-nitro-arginine methyl ester--infused groups, and the percentage of mortality was dose-dependent. Approximately 8% of all pups were dead in the animals given 25 mg of L-nitro-arginine methyl ester per day. This number was significantly ($p < 0.05$) higher than that in vehicle-infused animals, 2.5% Table I. The mortality rate was 18% in animals receiving 50 mg/day, and this value was significantly ($p < 0.01$) higher than that in animals infused with 25 mg/day or vehicle only Table I. The mortality rates in animals receiving either 240 or 480 micrograms nitroglycerin per day were similar to those of vehicle-infused animals Table I.

To determine if nitric oxide inhibition during pregnancy produced proteinuria, we analyzed the aliquots of urine obtained on day 21 of pregnancy in animals receiving 50 mg/day. The concentration of protein (2.34 +-\0.47 mg/ml) in these animals was significantly ($p < 0.05$) higher than that of controls (0.63 +-\0.41), suggesting that L-nitro-arginine methyl ester treatment during pregnancy causes proteinuria.

Comment

In this study we describe the effects of an inhibitor of nitric oxide synthesis during pregnancy on blood pressure, fetal growth, parturition, and fetal death. Constant infusion of the nitric oxide synthase inhibitor L-nitro-arginine methyl ester from day 17 of pregnancy to term caused an elevation in systolic blood pressure, proteinuria, and fetal growth retardation and increased fetal mortality, without altering the day of spontaneous labor. The effects of L-nitro-arginine methyl ester were significant and dose-dependent. These results indicate that inhibition of nitric oxide synthesis during pregnancy produces signs similar to those of preeclampsia and suggest that an alteration in nitric oxide synthesis may be one of the major problems responsible for preeclampsia.

Infusion of L-nitro-arginine methyl ester caused increases in systolic blood pressure, providing strong evidence that its action is caused specifically by the inhibition of nitric oxide synthesis. The elevations in blood pressure we observed are similar to the results reported for nonpregnant rats [19,20]. However, in the majority of these studies the predominant animal model

used for examining the effects of nitric oxide inhibition on blood pressure measurements were male rodents. Moreover, the administration of the nitric oxide synthesis inhibitors was through single and multiple bolus injections or through intravenous infusions of short duration (<10 hours). In one study [14] intravenous infusion of nitro-L-arginine for 8 hours was reported to increase mean arterial blood pressure in the female rat. This increase in blood pressure was higher during pregnancy compared with the postpartum period, indicating that nitric oxide may play a key role in the regulation of blood pressure during pregnancy. The current study indicates that chronic inhibition of nitric oxide production elevates blood pressure and causes a reduction in fetal weight. Thus this study highlights the significance of basal nitric oxide production, which may be critical for normal fetal development.

The above hypothesis is supported by the following studies. The in vitro release of nitric oxide by vascular tissue has been shown to increase during pregnancy in the guinea pig [8]. Also the decrease in blood pressure in pregnant spontaneous hypertensive rats has been shown to depend completely on endothelial nitric oxide release [15]. These studies indicate that nitric oxide may play an important role in the pathogenesis of preeclampsia. This is further supported by our study. Moreover, Van Buren et al [21] have suggested that nitric oxide may play a role in regulating uteroplacental blood flow in pregnancy and that L-nitro-arginine methyl ester treatment of late-term pregnant ewes leads to a decrease in uterine blood flow and an increase in blood pressure.

The mechanisms for the reduction in fetal weight in pregnant rats infused with L-nitro-arginine methyl ester are not clear at present. However, we believe that inhibition of the tonic release of nitric oxide increased vasoconstriction of vessels for placental circulation, thereby reducing the blood flow, could be responsible for fetal growth retardation. In support of this is the generally accepted concept that preeclampsia and hypertension accompany reduced fetal perfusion and the decrease in fetal growth.

Although several causal factors have been proposed for preeclampsia, there has been no consensus in this respect. Increased synthesis of PGI_2 over TxA_2 has been proposed as a possible mechanism for vasodilation and for the reduced sensitivity of the maternal vascular system to pressor agents in normal pregnancy [4,5,6,7]. The concept that an imbalance in the ratio of TxA_2 to PGI_2 in favor of the vasoconstrictor action of TxA_2 is responsible for major clinical symptoms of preeclampsia has been disputed, because inhibitors of cyclooxygenase failed to alter pressor responsiveness in the rat [22]. Moreover, there is a growing body of evidence that indicates that nitric oxide produced by the endothelium may play a critical role in the local regulation of blood pressure. Increased basal and acetylcholine-induced nitric oxide release and enhanced relaxant effect of acetylcholine in vascular tissue, together with significant increases in plasma and urinary levels of cyclic guanosine 5'-monophosphate [22] during gestation, supports a role for nitric oxide in blood pressure regulation in the pregnant female.

There is some evidence to show that treatment with nitric oxide donors inhibits uterine contractility during pregnancy, [23] and therefore endogenous nitric oxide might be involved in maintaining uterine quiescence during pregnancy. However, L-nitro-arginine methyl ester--infused pregnant rats delivered spontaneously at term, without altering the length of pregnancy Table I. Moreover, nitroglycerin-infused pregnant rats also delivered spontaneously at term without modulating the length of pregnancy Table I. This indicates that the nitric oxide donor was unable to inhibit spontaneous labor and that it did not extend the length of gestation. These results show that nitric oxide manipulations alone are not sufficient to alter the timing of labor and delivery.

Several animal models have been proposed for studying preeclampsia, such as the use of surgical reduction of placental perfusion, food deprivation, and treatments with various drugs in different animal species, including nonhuman primates [24,25,26]. However, the reproducibility of these models has not been very encouraging. In addition, very little work has been presented on the use of these models for applications to test various therapeutic strategies. Subcutaneous infusions of L-nitro-arginine methyl ester to pregnant rats produces symptoms similar to preeclampsia, and therefore we believe this can be used as an effective animal model for further studies of preeclampsia.

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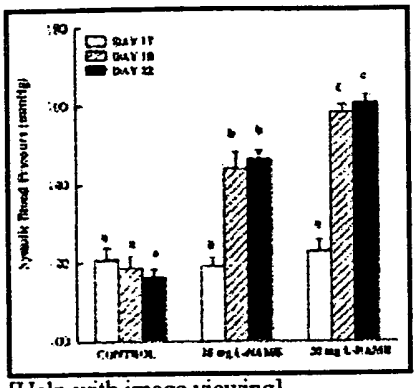

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Figure 1. Effect of L-nitro-arginine methyl ester (L-NAME) on systolic blood pressure of pregnant rats. Blood pressure was measured on day 17, before the osmotic minipumps were subcutaneously implanted, day 18, and day 22 of gestation. Animals received 25 or 50 mg of L-nitro-arginine methyl ester per day in saline solution or saline solution only (Control). Bar, Mean +-\SEM for 8 to 10 rats per group. Bars with different letters at top differ significantly ($p < 0.01$)

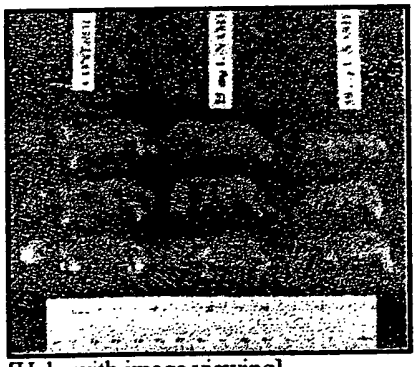

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Figure 2. Photograph depicting three representative pups of each group delivered by pregnant rats receiving 25 or 50 mg of L-nitro-arginine methyl ester (L-NAME) per day or saline solution only (Control). L-nitro-arginine methyl ester was infused subcutaneously through osmotic minipumps implanted on day 17 of gestation. Note size of pups was reduced by L-nitro-arginine methyl ester infusion in a dose-dependent manner

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Preterm Birth Prevention: Where Are We?

[Transactions Of Agos]

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Panel presentation, presented by invitation at the Eleventh Annual Meeting of the American Gynecological and Obstetrical Society, Hot Springs, Virginia, September 10-12, 1992.

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Outline

Graphics

Abstract

OBJECTIVE: The purpose of this study was to review the current approaches to preventing preterm delivery.

STUDY DESIGN: The problem of preterm birth was assessed by reviewing the different components that play a role in preterm birth prevention, excluding infection, antibiotic treatment, and tocolytic treatment.

RESULTS: Prevention of preterm labor must initially discriminate those at risk. Positive predictive values of various approaches are currently not adequate enough to warrant intervention. Prevention modalities, in part because of poor prediction, are mostly unproved.

Accurate diagnoses of preterm labor remains difficult and confuses analyses of tocolytic agents. Cervicovaginal fetal fibronectin, perhaps in combination with cervical evaluation, shows promise. Early detection programs remain controversial, but most reviews indicate that daily patient contact with high-risk patients gives cause for some optimism. Antenatal maternal glucocorticoid treatment at specific gestational ages improves neonatal outcome.

CONCLUSION: The incidence of preterm birth is rising in the country. However, improved definition of the various components of the problem has provided an improved understanding of the problem. There is a new continuing effort and a search for new and innovative ways to address this vexing national problem. (AM J OBSTET GYNECOL 1993;168:1223-30.)

Key words: Preterm labor: prediction, prevention, diagnosis, and early diagnosis

An optimist is a person who sees a green light

everywhere, while the pessimist sees only the red

stoplight.... The truly wise person is colorblind.

\\\Albert Schweitzer

The past 25 years has been a time of significant advances in perinatal medicine and in our understanding of reproductive processes. Unfortunately, although we have made progress in improving the outcome of pregnancies complicated by erythroblastosis fetalis, intrauterine growth restriction, diabetes mellitus, certain fetal malformations, etc., we have also not seen any significant reduction in the incidence of preterm birth. Throughout this time there have been green lights and red lights as we have sought the final truth in trying to deal with the complication of pregnancy. As the relative magnitude of the problem of preterm birth has increased while some of these other factors potentially complicating reproduction have been dealt with more successfully, the question is whether any progress at all been made in dealing with preterm labor and delivery. I think it is prudent to stay that, in relation to preterm births, we have yet to reach that color blind state that Dr. Schweitzer spoke of. Should we begin seeing the green or the red lights, and what are some of the key road blocks that we need to address?

The problem

There is certainly some cause for pessimism when looking at the incidence of preterm birth in the United States over the past decade. Data derived from birth certificate information reveal that as the number of births has increased in our country so has the proportion of those births ending before 37 weeks of gestation also increased. The incidence has risen from 9.4% in 1981 to 10.6% or 10.7% in 1989 (depending on a change in 1989 recording procedures) [1] Figure 1. Racial differences continue to be dramatic, with 8.8% of white and 18.9% of black births being preterm in 1989, although

the incidence of preterm birth has risen in both races in recent years.

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Figure 1. Incidence of preterm birth in the United States, 1981 through 1989. (Source: National Center for Health Statistics [1].)

A large multicenter trial (1983 to 1986) in which gestational age was meticulously determined in 33,401 pregnancies revealed the rate of preterm delivery, between 20 and 36 completed weeks of gestation, to be 9.6%, giving some additional credence to the national birth certificate --derived data [2]. Some 83% of the neonatal deaths in that study occurred in pregnancies ending before 37 completed weeks of gestation, and 66% of neonatal deaths were derived from those pregnancies ending at <29 weeks. Gestational age provides better prediction of survival before 29 weeks, whereas birth weight tends to be more important thereafter. Before 29 weeks male infants had a twofold increase in mortality and twin infants a threefold and fourfold increase in comparison with singleton female infants.

Because survival exceeds 90% by 30 completed weeks of gestation and 90% of otherwise uncomplicated preterm births occur between 30 and 36 weeks of gestation, neonatal morbidity issues assume paramount importance during this latter period of preterm gestation [3]. Significant improvement occurs by the extension of otherwise uncomplicated pregnancy by 1 week until after 36 completed weeks for the incidence of neonatal respiratory distress syndrome and until after 32 weeks for neonatal patent ductus arteriosus and necrotizing enterocolitis. High-grade intraventricular hemorrhage diminishes rapidly after 27 weeks and is virtually absent after 32 weeks.

The first issue in the attack of any medical disorder is the definition of the problem so that focus can be brought to bear on the disorder or its subsets. After centuries of collecting statistics on birth weight outcomes, rather than gestational age, I think we now have data based on what the obstetrician uses in decision making, namely gestational age.

We have learned that in assessing the impact of a new tocolytic agent on neonatal mortality caused by preterm birth, the gestational age to focus on is <30 weeks, not 30 to 36 weeks. It is noteworthy that in the recent, largely negative Canadian tocolytic-placebo trial that it was only in the 24 to 27 week category that there was a trend to lower infant mortality (11.5% vs 18.7%; differences, 95% confidence interval - 19.1% to 4.7%) [4]. The fact that only 1% to 1.5% of births occur in this early window of preterm

gestation, wherein mortality is a major outcome variable that can be addressed, makes design of tocolytic trials difficult if a decrease in mortality is the only point of concern.

An obstetrician faced with preterm labor between 30 and 36 weeks now has good information on the incidence of neonatal morbidity upon which management decisions can be made. Investigators, knowing the incidences of preterm delivery of morbidity by gestational age, can also now focus on particular time frames in which to determine the potential impact of any new specific therapeutic approach.

Preterm labor and delivery is often thought of as a social disease rather than a medical disorder. There has been a tendency for obstetricians not to focus on the disorder, but rather to let our legislators fix our social ills. A review of two of our leading peer review journals, the AMERICAN JOURNAL OF OBSTETRICS AND GYNECOLOGY and Obstetrics and Gynecology, reveals a total of only seven articles and two brief communcations on the subject of preterm birth for 1970 and 1971, supporting the view that preterm birth was then not of high priority for our specialty. A similar review for 1990 and 1991 reveals a total of 118 articles and eight brief communications on the subject Table I. This review did not consider the addition of many new journals that now also carry articles on preterm birth. It would appear that the specialty has at least begun to recognize and act on the issue of preterm birth, a necessary second step after defining the problem. However, it is necessary for physicians in our society to not only address the medical aspects of preterm birth but to collectively voice this concern publically and with persistent force so that the local, state, and national individuals responsible for public health policy will take new initiatives to decrease social ills that are associated with preterm delivery. National programs that have included a reorientation of the use of public funds, such as that in France, have shown great promise [5]. However, it is to be noted that 3 years elapsed before a beneficial effect was noted in the French experience, indicating that any trial of a new approach should be designed to cover at least 4 years if social and behavioral changes are part of the intervention [5]. Global societal problems such as the birth rate for teenagers, which rose almost 20% between 1986 and 1989, [1] or the increasing use of illicit drugs such as cocaine and its attendant incidence of preterm births of 21% to over 50%, [6,7] are but two examples of major societal problems that affect the preterm birth rate. We now recognize any trial of a new tocolytic agent should also entail drug screening for any patients entered into the trial, so that proper conclusions can be arrived at in the analyses.

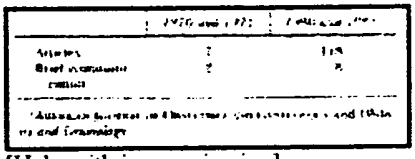

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Table I. Peer review articles on preterm birth

Society in general is not totally responsible for preterm birth nor for all the triggering mechanisms for initiating it. Assisted reproductive techniques have been of major assistance to thousands of childless couples, but these techniques are also associated with increased preterm birth rates. Nationwide data from some 25 units in Australia and three in New Zealand have been systematically collected over the past decade, totaling 3662 live births resulting from in vitro fertilization (IVF) and 1638 from gamete intrafallopian transfer [8]. Between 1979 and 1989 the incidence of twins with IVF and gamete intrafallopian transfer remained at about 19% to 20% and that for triplets at about 3% to 4%. It is too early to determine if the policy of transferring three or fewer embryos will result in a significant decrease of these higher-order births. The overall preterm birth rate throughout the decade remained at approximately 27% for both IVF and gamete intrafallopian transfer Table II. Although the incidence of preterm birth in twin and triplet pregnancies averages 55% and >95%, respectively, near expected spontaneous occurrences, the incidence in IVF singleton pregnancies also held steadily at approximately 17% over the past decade, and the incidence in gamete intrafallopian transfer singleton pregnancies has been approximately 15% from 1985 through 1989. Obviously those individuals responsible for leading our assisted reproductive techniques need to address this issue.

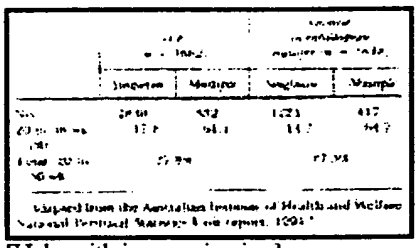

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Table II. Incidence of preterm live births in IVF and gamete intrafallopian transfer pregnancies, Australia and New Zealand, 1979 through 1989

Prevention of preterm birth

There are two potential approaches to any effort directed toward decreasing preterm deliveries. First is the potential for preventing preterm labor from being initiated and, second, the inhibition of the preterm labor process.

The first necessity in preventing initiation of preterm labor is knowing which patients are at risk for developing preterm labor or preterm premature rupture of membranes. Any prediction modality should have a high enough positive predictive value to warrant some sort of intervention or treatment and, second, enough sensitivity to affect national outcomes.

A number of risk scoring indices have been generated on the basis historic and current pregnancy factors. Unfortunately there have been few evaluations of these systems preformed in a prospective manner and without interventions [9]. However, even with that shortcoming the overall results

show positive predictive values of only approximately 15% to 30% and sensitivities of 35% to 60% [9,10].

Because the cervix participates in any preterm or term labor process, a number of recent studies of preterm birth have focused on the cervix. Questions as to whether a short cervix might predispose to ascending infection by interfering with the normal host-defense mechanism or whether cervical evaluations may predict preterm labor remain unanswered. Previous studies evaluating cervical dilatation have generally shown positive predictive values of approximately 25% at best [11,12]. Two more recent reports using a cervical score (cervical length in centimeters - cervical dilatation in centimeters) in multiple gestations have provided more encouraging results [13,14]. In these two studies 154 of 294 multiple pregnancies had preterm birth. The positive predictive value of a score <=0 ranged between 66% and 75% for all patients, and only two of 154 preterm births occurred within 1 week of a score of >=1 Table III. As the score became more negative, the interval to preterm delivery become shorter. The positive predictive value increased the earlier in the gestation that a score of <=0 was found, an important issue relative to potential reduction of neonatal mortality associated with preterm labor. It would seem that further study of this approach, including singleton gestations, is warranted to substantiate these encouraging results.

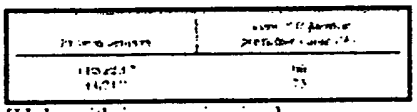

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Table III. Prediction of preterm delivery by cervical score (multiple gestations)

The introduction of endovaginal ultrasonography has led to further precise study of cervical length relative to preterm delivery [15]. Norms have been created from cross-sectional studies. Although the risk of preterm delivery is increased to 25% with a cervical length of <3.9 cm, the risk is only 35% at <3.4 cm. Further study, particularly longitudinal investigation, is needed before determining this modality is a discriminatory predictor of preterm birth.

A large number of biochemical assays have been assessed as predictors of preterm labor and birth and found to be lacking in efficacy. Recent interest has centered on a potential role for fetal fibronectin. Cross-sectional studies of 163 women with uncomplicated pregnancies delivered at term revealed that only 3% to 4% of cervicovaginal secretions were positive for fetal fibronectin between 21 and 37 weeks [16]. In another series of 117 preterm patients with intact membranes and uterine contractions, a positive fetal fibronectin test had a positive predictive value of 83% and a sensitivity of 82% for subsequent preterm birth in spite tocolytic therapy in some patients [16]. This information led to further longitudinal studies [17]. After excluding eight patients with indicated preterm births and three with

recurrent yeast vaginitis, there were 87 asymptomatic patients at high risk for preterm labor who had more than three samplings of cervicovaginal secretions for fetal fibronectin determinations between 24 and 34 weeks of gestation. A positive test had a positive predictive value for preterm delivery of 46%, a sensitivity of 93%, and a negative predictive value of 94%. These studies will need to be repeated in low-risk and high-risk populations before it can be determined if discrimination is adequate enough for intervention.

Uterine activity monitoring for prediction of preterm labor (in contradistinction to early detection of preterm labor) has provided positive predictive values of approximately 25% in the limited studies to date [18]. The wide variability and diurnal rhythms of uterine activity in individuals is likely to prevent good discrimination for this approach to prediction [19].

The concept of cardiovascular risk factors has become well ingrained in our society, and in many ways this is an educational system whereby individuals modify their lifestyles, habits, or actually receive drug therapy to reduce risks. It may be advisable to consider the same concept for preterm delivery risk, combining well established known risk factors such as smoking, poor nutritional status, previous preterm delivery, etc., with some biochemical tests such as fetal fibronectin. This risk approach could be used to address smoking cessation and stress reduction, treatment of asymptomatic bacteriuria, cessation of drug abuse, etc., and information regarding preterm labor, the subtle symptomatology of its early presentation, etc. This would certainly help to focus lay attention on the importance of all of these factors on preterm birth and also on reproductive outcomes in general.

Studies directed toward the prevention of preterm labor as a means to prevent preterm birth have mainly been done in twins wherein the incidence of preterm labor is approximately 40% to 50%. There have been four controlled trials assessing the role of in-hospital bed rest in twin pregnancies. None of the trials showed any benefit, and two indicated an increased incidence of preterm labor and preterm birth Table IV. The subject of decreased physical activity and modified bed rest out of the hospital in the home setting, with perhaps less stress and anxiety, will need to be addressed before this issue is put to rest. Numerous trials of prophylactic beta-adrenergic tocolytic usage with relatively low doses of medication have also not shown benefit in either singleton or multiple gestation [23]. Meta-analysis of five trials of prophylactic 17alpha-hydroxyprogesterone to prevent preterm labor have revealed that this agent is effective (odds ratio 0.50, 95% confidence limits 0.30 to 0.85), but these results were not associated with a decrease in perinatal mortality or respiratory distress syndrome [23] Table V. Further study of this approach is warranted.

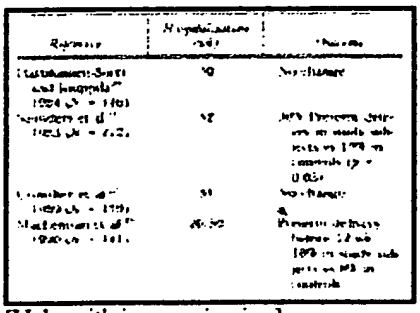

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Table IV. Four randomized studies of bed rest in hospital in twin gestation to prevent preterm delivery

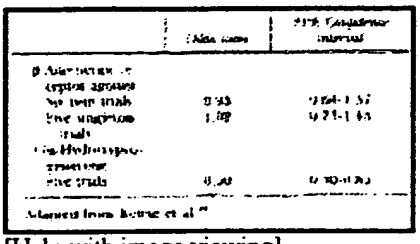

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Table V. Meta-analysis of randomized prophylactic drug trials for prevention of preterm labor and delivery

However, until there is a mechanism to accurately predict preterm labor we will have great difficulty in assessing any potential new therapeutic modality, and thus the first need is to be able to find an accurate discriminatory tool. We do not have any well established method of preventing preterm labor in 1992.

As indicated earlier in the discussion on tocolytic drugs, [25] a major problem in performing these trials has been in making the diagnosis of preterm labor. A meta-analysis of 12 randomized, controlled trials revealed a marked placebo effect with 27% to 89% of patients having delivery delayed by placebo treatment for 48 hours and 37% reaching term [24,26]. An error of 30% to 40% in the diagnosis of any disease obviously hinders an analysis of any intervention. A real need in our dealing with preterm birth is the ability to accurately diagnose preterm labor. In patients with intact membranes, regular contractions, and the cervix 1 to 2 cm dilated and at least partially effaced, 20% delivered at term with placebo therapy [27]. This type of information has led us to the necessity of evaluating the cervix and uterine activity in trying to diagnose preterm labor. As mentioned above, there was a positive predictive value of 83% of preterm delivery if fetal fibronectin was positive in cervical-vaginal secretions when patients presented with preterm uterine contractions [16]. Cervical dilatation at the time of entry was also found to be an independent contribution to final preterm birth, but there was no correlation between dilation and a positive test. It remains to be seen whether a combination of fetal fibronectin and cervical evaluation may improve the positive predictive value and sensitivity and specificity to 90% to 100%, but this new approach is encouraging. If we can differentiate false from true preterm labor, the evaluation of tocolytic drugs will be made much more accurately, and patients would not b

unnecessarily treated. The fact that 94% of patients with gross rupture of membranes by standard criteria also have a positive fetal fibronectin test is a potential confounding issue that needs sorting out [16].

The successful use of any tocolytic agent must depend on the early diagnosis of preterm labor, for if the cervix is >=4 cm dilated or the membranes are ruptured, tocolysis is rarely effective and the incidence of ascending infection is probably higher. Preterm birth prevention programs have in general been designed to maximize the early detection of preterm labor, with the hypothesis being that earlier detection would lead to more patients being candidates for tocolysis at an earlier stage of preterm labor, thereby being more effective at inhibition. Unfortunately, the tocolytic aspect of most of these trials has not been well controlled. The largest multicenter trial, involving mainly low-income populations, showed no beneficial effect but with substantial heterogeneity of program effects between centers [28]. Overall, the results have been mixed with the better outcomes noted in well educated, middle-income populations [29,30,31,32]. The original trials used patient education and self-palpation as a means of early detection. This approach was then extended by the use of daily instrumental home uterine activity monitoring in high-risk patients and again the results have been controversial, [33,34,35,36,37,38,39,40] and we have not reached that color-blind state that Schweitzer spoke of.

Although the important end-point of these types of approaches must be the prevention of preterm birth and neonatal morbidity and mortality, the first end-point must be the early detection of preterm labor. Only one trial has assessed whether monitoring by itself without nursing support is capable of improving early detection of preterm labor [38]. In this trial in which spontaneous preterm labor was diagnosed in 25% of the monitoreed patients and 24% of the control patients, the mean cervical dilatation was significantly less in the monitored group (1.4 vs 2.4, $p < 0.001$), and there were significantly more patients in the monitored group at each cervical dilation from <1 through 4 cm. This has in general been a feature of most of the reports. However, after this issue of early detection, there is considerably more controversy. Reviewers have pointed out the various methodologic deficiencies in the various trials and also stressed the difficulty in ascertaining the contribution of the uterine contraction and the daily specialized nursing contact. A Diagnostic and Therapeutic Technology Assessment panel of the American Medical Association concluded that "It has been shown that the system of home monitoring of uterine activity that includes daily nursing contact can lower the preterm birth rate in high-risk women...the relative contributions of the monitored data vs the nursing contact are under question" [39]. A National Institute of Child Health and Human Development and Bureau of Maternal and Child Health workshop summary stated, "In spite of these limitations the finding that the combined nursing and monitoring program has led to a substantial reduction in preterm births in these very high risk women remains a striking, perhaps unprecedented result" but went on to state that further randomized studies are needed [35]. A recent review by Grimes and Schulz [33] concludes, "To

date randomized controlled trials of home uterine activity monitoring published in peer review journals have not met accepted standards. Nevertheless, these and other unpublished trials suggest that home uterine monitoring lacks efficacy" [33]. One further article, critical of home uterine activity monitoring, went on to state, "In summary, there appears to be ample evidence from randomized trials that daily telephone contact with providers is effective in preventing preterm deliveries in a selected group of patients prospectively identified as being at risk for preterm delivery" [37]. It must be concluded at present that there is no consensus on the role that these programs play in preventing preterm birth; however, it is quite evident that there is considerable fervor and opinion on both sides of the coin and some optimism is obviously present.

Progressive preterm labor

Progressive premature labor continues to be a reality, because of infection, preterm premature rupture of the membranes, late presentation of patients, or ineffective tocolysis. At this point obstetricians try to aid the neonatal course of the fetus. An evaluation of 12 randomized, controlled trials of prenatal glucocorticoid therapy reveals a valuable decrease in the incidence of respiratory distress syndrome, with an odds ratio of 0.48 (95% confidence limits 0.40 to 0.58) Figure 2 [41]. A more marked effect (odds ratio 0.31) is seen if delivery can be delayed for 24 hours but not more than 7 days. In addition, data that were available in four of the trials revealed a decreased incidence of periventricular hemorrhage (odds ratio 0.44, 95% confidence limits 0.22 to 0.88) and from three trials a decrease in necrotizing enterocolitis (odds ratio 0.32, 95% confidence limits 0.16 to 0.64). Last, the important incidence of neonatal mortality is also reduced (odds ratio 0.59, 95% confidence limits 0.47 to 0.75). The analysis of these trials together also demonstrate no consistent effect of gender of the fetus. These meta-analyses have shown no consistent effect on the incidence of maternal or neonatal infection (with or without the presence of ruptured membranes) [41,42].

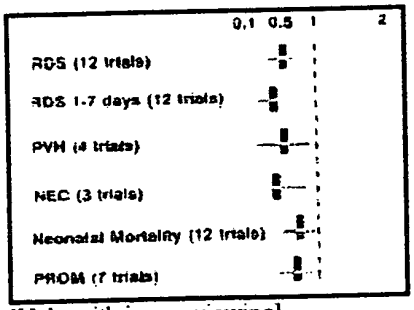

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Figure 2. Meta-analysis of effect of randomized trials of antenatal maternal glucocorticoid treatment on prevention of preterm neonatal mortality and morbidity. (Source: Crowley [41] and Keirse et al [42]) RDS, Respiratory distress syndrome; PVH, periventricular hemorrhage; NEC, necrotizing enterocolitis; PROM, premature rupture of membranes

These trials clearly have established a role for prenatal glucocorticoid therapy in preterm births occurring between 30 and 34 weeks of gestation. However, controversy has existed as to the role of glucocorticoids prenatally

when the membranes are ruptured. There has been considerable debate as to whether premature rupture of the membranes also decreases respiratory distress syndrome and whether any inherent decrease caused by rupture of the membranes might override the effect of prenatal glucocorticoid treatment. A meta-analysis of seven controlled trials with prenatal glucocorticoid after prelabor rupture of the membranes also shows a valuable effect on decreasing respiratory distress syndrome (odds ratio 0.55, 95% confidence limits 0.40 to 0.75) [42]. Another meta-analysis of five controlled trials also found a valuable effect, but the removal of one of the five reports with "the lowest quality" led to a conclusion of no effect [43]. The role of combining antibiotic treatment with glucocorticoid treatment prenatally is still not clear.

Because of the continuing high increase of respiratory distress syndrome and chronic lung disease (need for supplemental oxygen at 28 days of life) in newborns weighing <1500 gm, there have been searches for other approaches. Trials in experimental animals have shown that prenatal thyrotropin-releasing hormone, which crosses from the maternal to the fetal circulation and increases fetal triiodothyronone and prolactin, when combined with glucocorticoid therapy is more effective than glucocorticoid treatment alone in accelerating structural development and increasing lung compliance and surfactant production in these small fetuses [44]. The result of a recent randomized controlled trial of antenatal thyrotropin-releasing hormone and glucocorticoid treatment versus glucocorticoid treatment in gestations <32 weeks revealed that when a full course of treatment was given and delivery occurred within 10 days there was no difference in the incidence of respiratory distress syndrome (47% with combined treatment vs 58% in glucocorticoid alone) in newborns with mean birth weights <1500 gm [45]. However, chronic lung disease was significantly lower in the thyrotropin-releasing hormone--treated group (18% vs 44%), and there were significantly fewer cases of chronic lung disease or death (18% vs 38%). These preliminary findings are in accord with an unblinded study [46] and on early reports from another trial [47]. There have been no short-term adverse effects noted in the trials to date. A large Australian study is currently underway to determine if these encouraging results can be reduplicated. The possibility of affecting lung structure, as well as function, in a positive manner is most intriguing.

The role of infection in preterm labor and the use of antibiotics is dealt with elsewhere in this program [48]. However, it is clear that infection is the cause of some preterm labor but not all or even the majority [49]. It is also likely that other hormonal, immunologic, morphologic, or physical (distention) factors are playing a causal role in various incidents of preterm labor. Time does not permit an in-depth review of the cause of labor, and the reader is referred to recent reviews [50]. Although our knowledge of the mechanisms of parturition have increased significantly over two decades, the illusive final answer is still not in. In addition, approaches other than simple tocolytic therapy will probably become necessary as we recognize different specific causes that may be dealt with by specific approaches.

In summary, we have not been successful in decreasing the incidence of preterm birth. I do believe, however, that there is ample evidence that we are making some progress; we have defined the various problems with more rigor and we certainly understand more about the process. Difficulties in attaining consistent positive results in the various efforts to date need not lead us to put our heads in the sand and walk away. Rather, the results to date should encourage us to go forward with more effort and vigor, searching out new and innovative ways to overcome this vexing problem.

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Do Tocolytic Agents Stop Preterm Labor? A Critical and Comprehensive Review of Efficacy and Safety

[Transactions Of Agos]

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Panel presentation, presented by invitation at the Eleventh Annual Meeting of the American Gynecological and Obstetrical Society, Hot Springs, Virginia, September 10-12, 1992.
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Outline

Abstract

OBJECTIVE: Our aim was to determine the efficacy and safety of tocolytic agents currently used to treat premature labor.

STUDY DESIGN: We carried out a comprehensive review of tocolytic agents in the treatment of premature labor. Three hundred twenty-eight studies published between 1933 and 1992 were analyzed.

RESULTS: An analysis of randomized, placebo-controlled, clinical trials showed that magnesium sulfate is not better than placebo in the treatment of premature labor. beta-Adrenergic receptor agonists effectively stop premature labor for only 24 to 48 hours. Calcium channel blockers and oxytocin antagonists inhibit uterine contractions, but their rol in stopping labor is undefined. Prostaglandin inhibitors appear to be effective in treating

premature labor and have few adverse side effects.

CONCLUSIONS: The only tocolytic drugs that might be effective are the prostaglandin inhibitors. Tocolytic agents should be used only between 24 and 32 completed weeks of gestation. Magnesium sulfate should not be used to treat premature labor. Oxytocin antagonists should be used only in experimental clinical trials. Calcium channel blockers and beta-adrenergic receptor agonists inhibit uterine contractions but do not prolong gestation for longer than 48 hours. (AM J OBSTET GYNECOL 1993;168:1247-59.)

Key words: Premature labor, tocolytics, magnesium sulfate, beta-adrenergic receptor agonists, calcium channel blockers

Prematurity is the leading cause of infant morbidity and death. Thus, it is not surprising that physicians seek to prevent or stop premature labor. During the past 35 years, many pharmacologic agents have been used to inhibit labor. It is difficult to determine the efficacy and safety of tocolytic agents for many reasons. First, the cause of preterm labor is generally unknown. Therefore we cannot direct therapy to a specific cause. Second, in about 30% of patients with apparent preterm labor, uterine contractions cease spontaneously without treatment [1,2]. Finally, the diagnosis of preterm labor may be in error as much as 80% of the time [3]. By definition, labor should be diagnosed by the presence of regular uterine contractions combined with cervical dilatation and effacement. However, many investigations have based the diagnosis of preterm labor on contractions alone. The error in diagnosis with contractions used as the sole diagnostic criterion is 40% to 70% [4]. Despite the widespread use of tocolytic agents, the incidence of premature deliveries and the rate of low-birth-weight infants have not changed much in the last 40 years. Infants born prematurely still account for the majority of neonatal deaths [2].

The majority of patients diagnosed with preterm labor are not candidates for tocolysis, even if an ideal inhibitor were available [5]. Improvements in the perinatal mortality rate during the past two decades are probably due to advances in neonatal care rather than to the treatment of preterm labor.

In addition to problems in identifying true preterm labor, evaluation of tocolytic therapy is confounded by the variety of outcomes defined as "success". Criteria utilized as indicative of "success" of tocolytic therapy include temporary arrest of uterine contractions, delay of delivery for 24 to 48 hours, birth weight >2500 gm, and delay of delivery until 36 weeks.

Evaluation of the efficacy of tocolytic regimens is difficult because of the paucity of randomized, placebo-controlled studies. In addition, many studies have used combinations of drugs. Finally, tocolytic interventions may cause serious side effects in mothers and infants.

beta-Adrenergic agonists

Isoxsuprine was the first beta-sympathomimetic agent used to treat

premature labor, in 1961. Since then, orciprenaline, metaproterenol, salbutamol, albuterol, nylidrin, terbutaline, ritodrine, hexoprenaline, and fenoterol have been used. Ritodrine is the only drug currently approved by the Food and Drug Administration for treating premature labor.

Like the endogenous catecholamines epinephrine and norepinephrine, these drugs stimulate beta-adrenergic receptors in the uterus and other organs. There are two types of beta-adrenergic receptors in humans. $beta_1$ -Adrenergic receptors predominate in the heart, small intestine, and adipose tissue; $beta_2$ -adrenergic receptors are found in smooth muscle of the uterus, blood vessels, diaphragm, and bronchioles. beta-Adrenergic agonists affect smooth muscle cells through membrane-mediated binding to beta-adrenergic receptors that activates adenylate cyclase. This leads to an increase in intracellular cyclic adenosine monophosphate, which, in turn, initiates a series of reactions resulting in reduced intracellular levels of calcium and reduced sensitivity of the myosin-actin contractile unit to available calcium. The inhibitory effect on the uterus occurs even in the presence of oxytocin. Continued exposure to beta-adrenergic agonists leads to desensitization. With prolonged exposure, the number of beta-adrenergic receptors decreases (down-regulation), further reducing the effect of the drug.

Numerous reports describe the use of beta-sympathomimetics in preterm labor. However, the majority of the articles do not describe randomized clinical trials. Most clinical trials did not use placebo or control groups; therefore their results must be questioned.

Unacceptable clinical trials. Thirty-six reports, published from 1965 through 1991, describe nonrandomized, controlled clinical trials. Other articles either describe retrospective studies or use retrospective control groups.

Additional studies are confounded by the use of multiple agents in drug regimens. Szczygielski et al (1988) compared salbupart to fenoterol. One hundred seventy-one patients were randomly assigned to salbupart or fenoterol for treatment of premature labor. The two drugs were found to be equally effective. However, the patients also received verapamil, diazepam, and promethazine, and the method of randomization was not disclosed. In a similar study, Stockhausen et al compared three different beta-sympathomimetics with each other: Th 1165, dilatol, and TV 399. The study was randomized, although the method was not stated. They treated the patients with up to 45 minutes of infusion but did not comment on outcome.

Richter (1978) compared 167 patients receiving one of three beta-adrenergic agonists: buphenine, ritodrine, and fenoterol. In addition, some patients in each group were treated with verapamil. The study was not randomized and outcome data were not broken down by individual treatment group. These data were published again by Richter and

Hinselmann in 1979. Treatment assignment was random, but the method of randomization was not noted. Success was determined by means of the "prolongation index"--the lag time gained by therapy (in any units divided by the gestational age at the start of therapy (same units)). They concluded that ritodrine alone was less effective than the other treatments.

Several randomized trials compared different beta-sympathomimetics in patients in preterm labor. None showed a reduction in infant mortality or morbidity. However, no study was large enough to have detected a difference in outcome. Three additional studies compared ritodrine and terbutaline in randomized trials. They enrolled 252 patients in preterm labor with intact membranes. There was no difference in success rates (defined as arrest of preterm labor for >72 hours) between ritodrine and terbutaline.

Two studies looked at complicated pregnancies. One studied 10 diabetic patients. The other studied ritodrine in multiple pregnancy with three or more fetuses. Neither used control groups.

Two other studies attempted to eliminate patients not in true premature labor by initially sedating all patients. Neither used a control group for patients requiring treatment with beta-adrenergic agonists. A nonrandomized, prospective, placebo-controlled study of salbutamol has been reported. A double-blind, placebo-controlled study randomized patients to two different doses of salbutamol. Placebo was compared to oral salbutamol only after initial arrest of premature labor.

Elimination of these studies leaves 20 randomized studies published from 1966 through 1992 that compare beta-sympathomimetic agents with controls [6,7,8,9,10,11,12,13,14,15,16,17,18,19,20,21,22,23,24]. Thirteen used ritodrine, three used terbutaline, and four used isoxsuprine.

According to Keirse et al, [25] some of these studies were either conducted or reported in a manner that precludes unbiased evaluation of the treatment given: Das [9] (treatment allocation not randomized), Castren et al [1] (method of randomization unclear), Csapo and Herczeg [11] (questionable method of randomization), and Penney and Daniell [17] (provided no data on outcome by treatment allocation).

Some studies [10,12,14,16,20,23] probably should not be considered when the efficacy of beta-sympathomimetic therapy is evaluated for the reasons outlined in the subsequent text.

Merkatz et al [16] combined a number of studies from 11 clinical centers in such a manner that the results are impossible to interpret. There were different protocols for dosage and route of drug administration and an inadequate number of controls (some of whom were treated with ethanol). Patients entered into the trial of oral therapy were all pretreated with intramuscular ritodrine without randomization and were included in the treatment group. Four unpublished trials were included. A double-blind

study evaluating ritodrine versus placebo in 30 patients with rupture of membranes found no difference between placebo and ritodrine in prolonging gestation for >24 hours [14]. In a study that purported to evaluate the efficacy of beta-sympathomimetics versus placebo in patients with ruptured membranes 34 women (31%) were excluded and 23% of the patients were treated with at least one additional drug (terbutaline or magnesium sulfate) [23]. Two additional studies were not published in peer-reviewed journals [10,20]; therefore their methods and results have never been closely scrutinized.

Another study compared ritodrine to ethanol. One hundred thirty-five women were randomized between the two treatments. Ritodrine was more effective than ethanol in the inhibition of labor for 3 days and increased the days gained from initiation of treatment to delivery. This study is also unacceptable because no placebo was used [12].

Acceptable trials. Elimination of the flawed randomized studies leaves 10 placebo-controlled trials acceptable for analysis. Seven showed no benefit of beta-sympathomimetics over placebo in prolonging pregnancy [8,13,15,18,19,22,24]. The largest published study, by the Canadian Preterm Labor Investigators' Group, randomized 708 women to placebo or ritodrine. Assignment was stratified in four categories of gestational age. The study was conducted in six centers. The primary objective was to assess the effect of ritodrine on perinatal mortality. One of the secondary objectives was the effect of ritodrine on delay of delivery. The mean length of time from randomization to delivery was 27.8 +-\1.6 days in the ritodrine group and 24.5 +-\1.6 days in the placebo group (not significant). There was no difference in perinatal mortality [24].

Three studies show beta-sympathomimetics to be superior to placebo. Ingemarsson [7] compared terbutaline to placebo in 30 patients. Thirteen of 15 patients (87%) in the terbutaline group had labor arrested >7 days, compared with four of 15 patients (27%) in the placebo group. The author concluded that terbutaline is a safe and potent inhibitor of premature labor. A 1986 study randomized 125 patients to either ritodrine or placebo, although 26 patients (21%) were excluded after randomization. The mean number of days gained was 9.5 more in the ritodrine group. However, there was no difference between the two groups in the number of patients undelivered after 2 weeks [21]. The observation that 62% of the placebo patients were undelivered after 2 weeks, and 60% reached a gestational age of 36 weeks suggests that many of these patients were not in premature labor.

Wesselius-De Casparis et al [6] compared ritodrine to placebo in 91 patients undergoing 109 treatments for premature labor. They reported on the outcome in 81 treated cases: 43 patients assigned to ritodrine and 38 patients treated with placebo. There were patients with ruptured membranes in both groups. These patients were described separately. Twenty-seven of 35 patients (77%) with intact membranes were delivered after the treatment

period (up to 8 days, including oral therapy) and 17 of 33 (48%) in the placebo group had delivery delayed beyond the treatment period. Treatment with ritodrine for 1 week was sufficient to arrest premature labor during the treatment period and for some time beyond but not prolong pregnancy to term.

Side effects. These agents cause many unwanted effects because beta-adrenergic receptors are present in multiple organ systems. The cardiovascular system is most often involved. However, effects are also seen on the pancreas, kidney, gastrointestinal tract, and liver. The most frequently observed maternal symptoms are nausea, vomiting, tremor, and palpitations. Women also experience headache, thirst, restlessness, and chest pain.

Cardiovascular. The most common effects on the cardiovascular system are increases in heart rate, systolic blood pressure, pulse pressure, stroke volume, and cardiac output. There is a concomitant decrease in diastolic pressure and peripheral vascular resistance. Cardiac output can increase up to 60% over baseline levels. Mean arterial pressure does not change significantly. Cardiac arrhythmias have been reported. The most common is supraventricular tachycardia; other arrhythmias include atrial fibrillation, premature atrial contractions, and ventricular ectopy.

Increased heart rate and myocardial contractility can predispose to myocardial ischemia. Coronary artery perfusion is decreased as a secondary result of a decrease in diastolic blood pressure and diastolic filling time. These effects can cause micronecrosis of the myocardium. The electrocardiogram changes most frequently observed are transient ST segment depression and inverted T waves. These changes, which may be present in up to 76% of women treated with ritodrine, are often not associated with symptoms. They may be caused by relative hypoperfusion of the subendocardium. Others, who found no changes in cardiac enzyme levels, concluded that the electrocardiogram findings do not indicate significant myocardial damage. The electrocardiogram findings, which usually resolve with discontinuation of therapy, may not be due to myocardial ischemia, but rather to electrolyte imbalances.

Pulmonary. Pulmonary edema occurs in up to 5% of patients treated with beta-sympathomimetics. Pulmonary edema occurs with and without concurrent glucocorticoid therapy. Many cases are secondary effects of fluid overload resulting from the antidiuretic effect of high doses of beta-sympathomimetics. Fluid overload can also be a secondary result of excessive administration of intravenous fluids.

Plasma renin and arginine vasopressin are increased during infusion of beta-adrenergic agonists. This increase is associated with sodium and water retention, which predisposes to pulmonary edema. Pulmonary edema is more common in twin gestations. Infection may also play a role in the development of this complication. In the absence of underlying disease, most cases of pulmonary edema can be attributed to intravenous fluids and

to ignoring signs of fluid overload.

Metabolic. beta-Sympathomimetics increase maternal blood glucose about 40% with a concurrent increase in insulin secretion. The rise in glucose levels is even more pronounced in diabetes, probably because stimulation of glucagon secretion results in gluconeogenesis and glycogenolysis. Insulin levels rise as a result of hyperglycemia and also from direct stimulation of beta-adrenergic receptors in the maternal pancreas. Insulin release precedes the onset of hyperglycemia. The effect is heightened by concomitant administration of corticosteroids. beta-Adrenergic agonists also induce lipolysis, which increases acidic metabolites and can lead to severe metabolic acidosis in diabetic patients.

Serum potassium concentrations decrease rapidly at initiation of treatment with beta-sympathomimetics. The potassium concentration is usually 0.6 to 1.5 mEq below pretreatment levels. This decrease in serum levels is probably due to a net flux of potassium from the extracellular to the intracellular space. The hypokalemia is transient; replacement therapy is not indicated. Levels normalize within 24 hours of initiation of tocolysis.

Other effects reported include maternal transaminase elevations, paralytic ileus, myotonic muscular dystrophy, postpartum cardiomyopathy, respiratory arrest caused by muscle weakness in a patient with myasthenia gravis, acute cutaneous vasculitis, allergic dermatitis, hypertensive crisis, cardiac failure, agranulocytosis, cerebral ischemia, second-degree heart block, massive vulvar edema, adult respiratory distress syndrome, severe hemolytic anemia, and maternal death.

Fetal side effects. beta-Sympathomimetics rapidly cross the placenta. Stimulation of beta-adrenergic receptors in the fetus probably evokes the same responses as in the mother. Cardiovascular effects include fetal tachycardia, increased cardiac output and redistribution of fetal blood flow, increased thickness of the fetal ventricular septum, neonatal supraventricular tachycardia, myocardial ischemia, myocardial necrosis, hydrops, and hypoglycemia and hyperinsulinemia in the neonate.

Studies examining alterations in uteroplacental blood flow have been conflicting. Some report decreased blood flow, others increased uteroplacental blood flow, and still others no significant change. Alterations in blood flow are not associated with significant changes in fetal hemodynamics. The differences may be attributed to the duration of infusion, the drug used, concurrent use of other medications, and the method used to measure uteroplacental blood flow.

Data from the controlled clinical trials discussed in the preceding text show no difference in neonatal mortality or severe respiratory morbidity between infants born to mothers treated with beta-sympathomimetics and those born to mothers in the control groups.

Infants exposed to beta-adrenergic agonists in utero have been followed up from 1 to 9 years. There are no differences in developmental outcome between exposed infants and preterm controls.

Conclusion. There are no verified benefits to mother or fetus from long-term therapy with beta-sympathomimetics to arrest preterm labor. Chronic exposure may adversely affect the fetus. Maternal side effects are inevitable and can be life-threatening. There is no evidence that calcium antagonists minimize or prevent these side effects. Fluid overload should be avoided if these agents are used.

beta-Sympathomimetics appear to stop contractions for 24 to 48 hours. However, only three studies found them better than placebo in prolonging gestation for >48 hours [6,7,21]. Their use should therefore be confined to situations where attainment of a 24- to 48-hour delay is the goal. Because of potential serious adverse effects, there are few circumstances in which these drugs are indicated.

Magnesium sulfate

Magnesium inhibits myometrial activity in vitro and in vivo. Magnesium inhibits uterine contractions induced by calcium and transiently inhibits further calcium response.

The mechanism by which magnesium sulfate exerts its tocolytic effect is unknown. Presumably, myometrial contractility is depressed by modulating calcium uptake, binding, and distribution in smooth muscle cells. In high concentrations magnesium blocks calcium influx by competing for calcium binding sites on the cellular membrane. Magnesium activates adenylate cyclase and increases cyclic adenosine monophosphate, thus reducing intracellular calcium. Serum concentrations of 4 to 8 mEq/L appear to be necessary for reduction of myometrial activity.

Controlled studies evaluating the efficacy of magnesium sulfate in inhibiting preterm labor are scarce. A few studies have been placebo controlled [26,27,28].

One report concerned 31 patients who received intravenous magnesium sulfate, 31 patients who received alcohol, and 9 patients who received dextrose. Control of premature labor was considered successful if contractions were stopped for 24 hours. By this criterion the success rate of magnesium sulfate was 77%. The rate of success was inversely related to the degree of cervical dilatation [26]. The study has several flaws. Only the magnesium and ethanol groups were randomized; the dextrose group was selected from the general population. The dextrose group was too small to demonstrate statistical significance and the magnesium sulfate dosage was inadequate. In view of the small number of control patients and the absence of a fully randomized protocol, the study failed to demonstrate any efficacy from magnesium sulfate.

A randomized study compared magnesium sulfate, terbutaline, and 5% dextrose for labor inhibition. The study population consisted of 54 patients between 26 and 34 weeks of gestation. Success was defined as delay of delivery for at least 48 hours. The study showed no significant differences among the three treatment groups in delay of delivery for at least 48 hours [19].

One hundred fifty-six women in preterm labor between 24 and 34 weeks' gestation were randomly assigned to receive either intravenous magnesium sulfate or no tocolytic therapy. Magnesium sulfate therapy had no effect on duration of gestation, birth weight, neonatal morbidity, and perinatal mortality [27].

Two randomized studies, neither placebo controlled, compared the tocolytic efficiency of magnesium sulfate against that of beta-adrenergic agonists. The first compared intravenous magnesium sulfate and terbutaline in a randomized study of 29 patients. Successful treatment was defined as arrest of labor for 24 hours. Magnesium sulfate and terbutaline were equally effective [28].

In the second study 70 patients were randomized to either magnesium sulfate or intravenous ritodrine hydrochloride. The mean dosage required to achieve tocolysis in the magnesium sulfate group was 4.5 gm/hr, one of the highest doses reported in the literature. Magnesium sulfate and ritodrine delayed delivery at least 72 hours in 88% and 79% of patients, respectively [29].

A study involving 111 patients (divided into three groups) used magnesium sulfate intravenously and continuously for >10 days. The drug had to be discontinued because of side effects in 7% of the patients in each group.

Dual-agent tocolysis after failure of single-agent therapy has also been reported. Twenty-three patients were treated with a combination of magnesium sulfate and ritodrine or terbutaline. Delivery was delayed for >=48 hours or more in 60.9% of patients, but pulmonary edema developed in 22% of the patients.

Side effects. When magnesium sulfate therapy is maintained in the nontoxic range, maternal side effects are few. Nausea, vomiting, ileus, visual blurring, diplopia, headaches, weakness, lethargy, shortness of breath, pulmonary edema, alterations in calcium metabolism, and urinary retention have been reported. Hypermagnesemia can occur in the presence of impaired renal function. Excessive levels of serum magnesium have been associated with respiratory depression, subendocardial ischemia, cardiac arrest and death.

Magnesium sulfate crosses the placenta. Fetal plasma concentrations are comparable to those in the mother. Neonatal hypotonia and drowsiness have been reported. Bony abnormalities and congenital rickets in the newborn

have been associated with magnesium sulfate infusion for tocolysis.

Conclusion. The large experience with magnesium sulfate in the treatment of preeclampsia coupled with its potential tocolytic effect suggested that it might be a useful agent to inhibit labor. Although magnesium sulfate inhibits spontaneous and oxytocin-induced uterine activity in vitro, safe dosages of magnesium sulfate are ineffective in preventing preterm birth.

Oxytocin antagonists

Labor, term or preterm, is associated with an increase in myometrial oxytocin receptors. Myometrial oxytocin receptors increase twelvefold at term, compared with those at 13 to 17 weeks of gestation, and also increase in women who are delivered preterm. Theoretically, oxytocin antagonists might provide effective tocolysis, because systemic side effects would be minimal because of organ specificity.

Several studies describe the effects of oxytocin antagonists in vitro. One investigated the ability of 1-deamino-2-D-Tyr(0-ethyl)^4 -Thr sup 8 -Orn-oxytocin to inhibit binding of oxytocin in decidual and myometrial membranes of the rat, guinea pig, and human. Decidua and myometrium of all three species bound tritium-labeled oxytocin with high affinity, and 1-deamino-2-D-Tyr(0-ethyl)^4 -Thr^8 -Orn-oxytocin completely displaced labeled oxytocin from binding sites in all tissues. Another examined a synthetic oxytocin antagonist (beta-mercapto-beta,beta-cyclopentamethylene proprionic acid, D-Trp^2 -Ile^4 -Arg^8)-vasopressin. This compound inhibited oxytocin-induced contractions in the nonpregnant rat uterus, in vitro and in vivo. It also inhibited milk letdown in the lactating rat, disrupted labor in the rat, and inhibited the in vitro response to oxytocin of myometrium obtained from women. Four oxytocin analogs, 1deamino-2-D-Tyr(0-ethyl)^4 -Val^8 -Orn-vasotocin, 1-deamino-2-D-Tyr(0-ethyl)^4 -Thr^8 -Orn-vasotocin, 1-deamino-2-L-Tyr(0-ethyl)^4 -Thr^8 -Orn-vasotocin, and 1-deamino-2-D-Trp^4 -Thr^8 -D-Arg-vasotocin, displaced oxytocin and arginine vasopressin in myometrial membrane preparations from pregnant women at term.

Two reports describe the use of oxytocin antagonists in women in preterm labor. The first used 1-deamino-2-D-Tyr(0-ethyl)^4 -Thr^8 -Orn-oxytocin in 13 patients. Uterine activity was inhibited in all patients. However, 10 patients received subsequent therapy with beta-sympathomimetics, and three (23%) were delivered preterm [30]. A second study used intravenous infusions of 1-deamino-2-D-Tyr(0-ethyl)^4 -Thr^8 -Orn-oxytocin in 12 patients in premature labor. All patients were initially placed at bed rest for 2 hours and were treated if contractions persisted. Complete tocolysis was noted in six patients, partial tocolysis in three patients, and no tocolytic effect in three patients. The three patients in whom there was no effect were

less than 28 weeks pregnant and were subsequently treated with ritodrine. Ultimately, 7 of 12 patients were delivered preterm. No maternal side effects, fetal heart rate abnormalities, or complications with breast-feeding were attributable to treatment. No comments were made regarding neonatal morbidity or mortality [31].

Conclusion. These studies demonstrate that oxytocin antagonists inhibit myometrial contractility. These agents need to be studied in controlled clinical trials to determine efficacy and safety.

Prostaglandin inhibitors

Local prostaglandin production probably plays a role in cervical ripening and may modulate uterine activity in labor. Many centers use prostaglandin E_2 gel for cervical ripening. Both prostaglandin E_2 and prostaglandin F_{2alpha} are used for induction of labor in the second trimester of pregnancy.

Prostaglandins exhibit uterine effects in two ways. First, they enhance production of myometrial gap junctions. Second, prostaglandin F_{2alpha} stimulates the influx of intracellular calcium and the release of calcium from the sarcoplasmic reticulum. This increase in intracellular calcium leads to activation of myosin light chain kinase and subsequent muscle contraction. Elevated levels of prostaglandins in plasma and amniotic fluid have been demonstrated during normal human parturition. Levels are low or absent in serum and amniotic fluid of patients not in labor at all stages of pregnancy. Prostaglandin metabolites are significantly reduced in patients treated with indomethacin. They are also significantly higher in patients who are delivered preterm than in patients with prolonged gestation [32].

All prostaglandin synthetase inhibitors act by inhibiting the enzyme cyclooxygenase. This enzyme is found throughout the body and in high concentrations in the myometrium. Cyclooxygenase converts arachidonic acid into the first prostaglandin intermediate prostaglandin G_2. All subsequent prostaglandins are derived from this initial step. Aspirin causes irreversible inhibition of this enzyme by acetylation. Indomethacin competes with arachidonic acid for cyclooxygenase. Therefore it does not disrupt the enzyme. When indomethacin levels decrease, enzyme activity resumes. These drugs have antiinflammatory, antipyretic, and analgesic properties. They also suppress formation of prostacyclin and thromboxane A_2. Indomethacin, naproxen, and fenoprofen are more effective than aspirin as inhibitors of prostaglandin synthesis.

Nonsteroidal antiinflammatory drugs differ in chemical structures, mechanisms of action, and side effects. Therefore one cannot assume that an effect observed with a particular agent will be found with another. These drugs effectively inhibit contractility of the pregnant and nonpregnant myometrium. They are more effective than the beta-sympathomimetics. There has been no report of suppression of uterine contractions with

beta-adrenergic agonists after failed treatment with a prostaglandin inhibitor, but several studies show the opposite.

Interest in prostaglandin inhibitors began in 1973. A retrospective study showed that patients taking high-dose salicylates during pregnancy had significant increases in mean length of gestation, frequency of postmaturity, and mean duration of spontaneous labor [33]. In a second study patients taking long-term salicylates during pregnancy had a higher incidence of gestation extending beyond 42 weeks. Another study demonstrated that administration of oral aspirin or indomethacin prolonged the injection-to-abortion interval in patients undergoing midtrimester abortion with hypertonic saline solution. Finally, low doses of aspirin prolonged the injection-to-abortion interval in nulliparous patients undergoing midtrimester abortion with hyperosmolar urea and continuous oxytocin infusion.

The first report of the use of these drugs to stop premature labor was published in 1974. Treatment of 50 patients in premature labor with indomethacin delayed delivery >7 days in 40 patients (80%) [34]. Numerous subsequent studies have attempted to evaluate the efficacy of prostaglandin inhibitors in treating premature labor.

Unacceptable trials. Six studies described the use of various prostaglandin inhibitors other than indomethacin. None were controlled clinical trials.

Additionally, several studies using indomethacin were noncontrolled clinical trials. For example, in one study 68 patients were treated with indomethacin. However, only 64 patients were in premature labor. All patients responded to therapy. There were no serious neonatal adverse effects. There was no control group. In another study nine patients who continued to have uterine contractions after initial treatment with bed rest and isoxsuprine were treated with indomethacin. The only conclusion drawn is that indomethacin may slow uterine contractions. In a third study indomethacin was given to 29 women initially treated with bed rest and ritodrine. The data cannot be analyzed to determine clinical efficacy.

Polydrug treatment was used in two studies. In 1991, 86 patients in preterm labor were initially treated with hydration and sedation followed by magnesium sulfate if contractions persisted. If patients met inclusion criteria, they were randomized to indomethacin and ampicillin-sulbactam or placebo. However, the efficacy of the indomethacin cannot be determined because of magnesium sulfate and antibiotic administration. A similar study treated 250 preterm labor patients with terbutaline. If this drug was ineffective, patients were treated with intravenous ritodrine, magnesium sulfate, or both. If contractions persisted and the gestation was <33 weeks, they were given indomethacin. The results are uninterpretable because of the multiple tocolytic agents used.

Acceptable trials. In a few controlled studies indomethacin was used to

inhibit premature labor, but in conjunction with either ethanol or beta-sympathomimetics. Spearing (1979) treated 42 patients either with ethanol and indomethacin or with ethanol alone. This trial suggested that indomethacin plus ethanol delayed delivery more effectively than did ethanol alone [35]. Four trials used indomethacin in conjunction with ritodrine [36,37,38,39].

Two randomized studies compared indomethacin to another tocolytic. The first compared ritodrine with indomethacin. One hundred six patients were randomized. However, some patients in both groups were subsequently treated with magnesium sulfate, and all patients were placed on a regimen of oral terbutaline after initial therapy. Indomethacin was as effective as ritodrine [40]. Another study randomized patients with refractory preterm labor to either indomethacin or sulindac. Sulindac was as effective as indomethacin [41]. Neither study reported serious adverse effects.

A randomized trial compared indomethacin to intravenous ritodrine in 40 patients with premature labor and intact membranes. Delivery was delayed for >7 days in approximately 67% of patients in each group. However, 33% of the ritodrine patients and 27% of the indomethacin patients were treated with magnesium sulfate after failure of initial treatment. All were continued on a regimen of oral therapy, with a mean duration of treatment between 25 and 30 days. Eighty percent of patients receiving beta-sympathomimetics complained of side effects, and 24% discontinued treatment because of them. Side effects were minimal in the indomethacin group. The drugs were equally effective. There were three cases of primary pulmonary hypertension in infants in the indomethacin group, allegedly because of duration of treatment. The 11% incidence of oligohydramnios in the indomethacin group prompted discontinuation of treatment. There was a large difference in cost between ritodrine and indomethacin ($560 vs $33) [42].

Only two studies compared indomethacin with placebo in patients with preterm labor. Both used indomethacin as initial treatment and were doubly blinded. However, some women in both studies were given other tocolytics when initial treatment failed: 30% of the placebo group in Zuckerman et al [43] and 44% of the placebo patients in Niebyl et al [32]. Neither study reported adverse side effects related to the use of indomethacin.

Side effects. Prostaglandin inhibitors are not associated with serious adverse effects on mother or fetus. There were no major problems in the newborns of 297 women treated with indomethacin [44].

These compounds differ in chemical structures, mechanisms of action, and side effects. Common adverse effects include nausea, vomiting, diarrhea, heartburn, headache, dizziness, and allergic rash. More serious toxicity is manifested by thrombocytopenia, peptic ulceration, bleeding, and serious allergic reactions. In addition, prostaglandin inhibitors may mask signs of infection.

The main concern with this class of drugs is the potential for adverse effects on the fetus, particularly premature closure of the ductus arteriosus. Indomethacin is used to treat persistent patency of the ductus arteriosus in the preterm neonate. Clinical response in the preterm neonate is variable and not related to serum indomethacin concentration. Most studies demonstrate resistance of the ductus to closure at earlier gestational ages. Prostaglandin inhibitors cause constriction of the fetal ductus arteriosus in utero. The constriction is transient and usually abates after cessation of the drug. However, prolonged exposure to indomethacin may lead to persistent pulmonary hypertension and tricuspid insufficiency in the neonate.

Other fetal complications include impaired renal function with resultant oligohydramnios. Indomethacin has also been used to treat polyhydramnios and normalize amniotic fluid volume. This drug may be especially useful for treating preterm labor in patients with polyhydramnios. There is little evidence that indomethacin causes permanent renal impairment in the neonate; one case report documented a monozygotic twin gestation with polyhydramnios in which the mother was treated with indomethacin and the fetus had renal dysgenesis.

Conclusion. Although controlled trials are lacking in quality and numbers, it appears that indomethacin can delay delivery. More data are needed before the effectiveness of prostaglandin inhibitors can be fully assessed.

Calcium channel blockers

Calcium channel blockers inhibit spontaneous myometrial contractions and suppress prostaglandin- and oxytocin-induced uterine contractions in vitro and in vivo. The main site of action is the cell membrane, where influx of extracellular calcium through voltage-dependent calcium channels is inhibited. Verapamil, but not nifedipine, impairs atrioventricular conduction and can cause cardiac dysfunction.

The use of verapamil for treating preterm labor was first reported in 1972. Effectiveness of treatment could not be shown because dosage was limited after cardiovascular side effects. The first study using nifedipine to treat premature labor was reported in 1977. Ten patients in preterm labor were treated. Labor stopped in all patients. In a similar study of 20 patients 15 had delivery delayed for >3 days. A subsequent study documented delay of delivery in eight patients with chronic hypertension until after 38 weeks of gestation. Mean gestational age at entry was 30 weeks. The patients became normotensive during therapy.

Delivery was delayed for >48 hours in 9 of 13 patients treated with nifedipine. Ghirardini (1991) reported successful treatment of eight women in premature labor. All were delivered after 38 weeks' gestation. In another study uterine contractions were inhibited in 16 of 22 patients in preterm labor treated with nifedipine; 13 experienced undesirable side effects.

Two prospective, randomized studies compared nifedipine with ritodrine. The first randomly allocated 20 women to ritodrine, 20 women to nifedipine, and 20 women to no treatment. Success was defined as delay of delivery for 48 hours. Seventy-five percent success was achieved in the nifedipine group, compared with 45% in the ritodrine group and 29% in the placebo group [45]. This study has been criticized for possible selection bias, poor reporting of side effects, and the fact that 25% of the nifedipine group were subsequently treated with ritodrine [46].

A recent study randomized 33 patients to ritodrine and 33 patients to nifedipine. Delivery was postponed for 48 hours in 84% of the nifedipine group and 72% of the ritodrine group. Delivery was delayed for 7 days in 70% of women treated with nifedipine and 52% of those treated with ritodrine. Maternal side effects were more common in patients treated with ritodrine ($p < 0.01$). Fetal and neonatal outcomes were similar in the two groups [47].

Side effects. These drugs produce vasodilatation and decrease peripheral vascular resistance. Transient facial flushing is the most common side effect, but they can also cause nausea and headache. Maternal side effects appear to be less than with the beta-sympathomimetics [47]. Nifedipine potentiates the toxicity of magnesium sulfate by causing neuromuscular blockade. It also causes maternal hepatotoxicity. Although no serious fetal or neonatal side effects have been reported, these drugs may diminish uteroplacental blood flow.

Conclusion. Data regarding these agents are insufficient to support clinical use.

Comment

The rate of prematurity has not declined in this country despite the utilization of numerous pharmacologic agents for treating premature labor. There is no ideal tocolytic agent. Many drugs have been tested but seldom in randomized, controlled trials. The accuracy of the diagnosis of premature labor is suspect in many of the published trials, especially when some report success rates in excess of 80%.

It is difficult to determine the efficacy of individual drugs, because criteria for success vary from study to study. A sample size much greater than any reported in the literature would be required to show a significant reduction in prematurity and/or perinatal mortality.

Overall neonatal survival for gestations of 24 weeks' duration is approximately 17%, rising to 51% at 26 weeks and to 95% at 32 weeks of gestation [48]. During the interval from 24 to 32 weeks' gestation an additional week in utero significantly increases perinatal survival.

In our opinion tocolytic agents should be used only between 24 and 32

weeks of gestation. The goal of therapy ideally is to prolong gestation to or beyond 32 weeks, but a delay of even 1 week is significant. Premature labor should not be treated with pharmacologic agents after 32 completed weeks. This opinion is supported by a recent study of >20,000 deliveries at five tertiary centers between 1983 and 1986. Dating of pregnancies was meticulous. Gestational ages of the neonates were confirmed at delivery by Dubowitz score. The incidence of necrotizing enterocolitis, patent ductus arteriosus, grade III and IV intraventricular hemorrhage, and sepsis markedly decreased after 32 completed weeks of gestation and virtually vanished after 34 completed weeks. The number of days of mechanical ventilation for respiratory distress syndrome and newborn hospital stay were also significantly reduced after 32 weeks [49]. Therefore one cannot justify pharmacologic treatment of premature labor after this gestational age.

We conclude that the only drugs that might be effective in treating premature labor are the prostaglandin inhibitors. These agents pose little risk to the fetus before 32 completed weeks of pregnancy. Oxytocin antagonists have never been tested against placebo and should be used only in experimental clinical trials. Magnesium sulfate is no better than placebo and has potential serious adverse effects in mother and fetus. It should not be used to treat premature labor. Calcium channel blockers and beta-sympathomimetics inhibit uterine contractions but do not decrease prematurity or perinatal morbidity, nor do they prolong gestation for >48 hours. Furthermore, mother and fetus are placed at substantial risk for side effects.

Discussion

Dr. Howard Jones III, Nashville, Tennessee. It seems to me that we have an ever-increasing group of working women in our residency programs who probably fit into this "active" category. What is your policy and what should be the policy for program directors for women residents in obstetrics and gynecology during pregnancy? Do they have a problem with premature labor and delivery, and what should we do about it?

Dr. Creasy. Dr. Cefalo, you're aware of a study that was done in your institution on this issue. Would you like to try to give us some counsel?

Dr. Robert C. Cefalo, Chapel Hill, North Carolina. It was a retrospective study on house staff versus other professionals who were in more sedentary occupations but at the same socioeconomic level, and it was found that there was a significant increase in preterm birth in the house staff working long hours.

Dr. Vern Katz of our group followed that up with a study of catecholamine excretion during working and off-time hours and found that physicians, house staff, and faculty had elevated catecholamine excretion during working hours.

I can't answer Dr. Jones' question, but we're very concerned about this effect of long hours of standing without any rest. So I concur that it's a real problem.

Secretary Scott. Dr. Pauerstein, did you say you actually have discontinued all use of tocolytics in your department?

Dr. Pauerstein. No, would that the chairman had such power. We did use magnesium sulfate, and that's one drug that I am certain is of no value, so we are no longer using it. I think it's going to take me a little while longer to do anything more definitive than that.

Secretary Scott. I would be surprised if there are many academic departments that don't use them. Maybe I'm wrong.

Dr. Kirschbaum. I think you're right. Objectively, there's no point in our continuing the use of tocolytics, in my view at least. I did see some benefit from their use during the time that I was at the University of Southern California. When 30 women are in labor and 10 of them have what seems to be preterm labor, it's nice to have contractions stop so the residents and staff don't have to spend all night examining these patients. I think that's the only value I've ever seen.

Secretary Scott. I wouldn't argue against that. I think the only evidence is that labor will stop long enough for the patient to get to a hospital with a newborn intensive care unit.

Dr. Pauerstein. I think that's about it, but the difficulty is once a practice is established, it takes an awfully long time to get it stopped.

Secretary Scott. The other question I had related to what Dr. Simpson said. Perhaps the only way we'll get at this problem is if there is some sort of a biochemical marker. Is there a possibility that there will be a biochemical test that the patient is at risk for preterm labor or is truly in preterm labor and at risk for delivering early? We need to try to do something.

Dr. Creasy. I don't think we can say that fetal fibronectin is the marker that can be used for prediction. Dr. Lockwood is doing a study at the present time in low-risk patients, and I understand the results are very promising, but that's all I can tell you. I think its role may well be to help us making that diagnosis of premature labor.

Dr. Kirschbaum. Dr. Lockwood's interest in fibronectin is as part of the extracellular portion of connective tissues. It appears in the general circulation in instances in which there is widespread endothelial damage.

Dr. Lockwood is interested in this particular identity because he believes, as he reported in the American Journal of Pathology (1991;138:T37), that oncofetal fibronectin plays a role in trophoblast attachment to the decidua.

Whether that's true is uncertain, but he demonstrated a relationship of some predictive strength for preterm labor in its appearance in the cervix. The next step is to act on it and see whether it improves results.

Dr. Creasy. The other real conundrum with fetal fibronectin is that it was originally designed as a test for premature rupture of the membranes, and its efficacy in doing that is much more than 90%. So the question comes up as to what do you do when you have a patient with positive test results? Is that for ruptured membranes or is it for premature labor?

Dr. Pauerstein. I would like to make one sort of general comment or observation. When cardiovascular disease was compared to premature birth, in both columns one thing, family history, was left out. As you read the literature, it's kind of amazing, but it seems very much that the economic condition of the mother when she was in utero is a big predictor of her having a premature birth.

If you go back to the Aberdeen birthday study that Sir Dugald Baird published, the most important predictive factor was the mother's father's social class. There has most recently been a study, I believe in The New England Journal of Medicine, just a very short time ago, which also suggested that the grandmother's history, socioeconomic and prematurity, had a strong impact on next-generation premature births.

I think we have to look at this as a very complex problem. Everybody laughed when Dr. Simpson showed the prematurity gene, but I'm not sure that that's not part of what's operative.

Dr. Kathy Nelson, Lexington, Kentucky. I was particularly interested in what Dr. Creasy had to say about the fact that despite all the efforts and energy that have gone into trying to intervene to decrease preterm births, over the last several decades there really hasn't been any substantial decline. Yet he pointed out that there have been numerous social and other things that happen, including drug use, worsening socioeconomic status, and more women in the work force, which, in fact, you would think might ultimately increase the number of preterm births if what we were doing was not having an effect on the baseline.

My observation is, perhaps if a particular risk factor was looked at more specifically, you could actually measure changes in something that we've done over the last decade to decrease that particular risk factor. You could say, for women with twins, we have done something that has influenced the preterm delivery for that particular category. I just wanted to know if you had a comment.

Dr. Creasy. My comment was based on the overall national incidence. There certainly have been programs that have been successful in their particular populations in decreasing preterm deliveries, and that is why I continue to use beta-adrenergic drugs because we have been successful whereever I've

been in decreasing the incidence of premature birth in our populations. So I will continue using the beta-adrenergic receptor agonists. I'm not going to stop them, to answer Dr. Scott's question.

Dr. Kirschbaum. Dr. Nelson, your question has to do with judging effectiveness against a shifting baseline. Those of us who are not Republicans believe that some people have gotten a lot worse and some people have gotten a lot better in socioeconomic status in recent years.

Dr. Guy M. Harbert, Charlottesville, Virginia. I just can't let Dr. Pauerstein get by with doing away with magnesium sulfate. We aren't going to eliminate it in Charlottesville. This has been somewhat of an extremeely legalistic conference. Dr. Creasy thinks screening doesn't help anything.

But before Dr. Scott goes back to Utah and does away with magnesium sulfate, has anybody else looked at what we are doing? With the preterm birth prevention programs at the University of Virginia, the instance of preterm births or low-birth-weight births did not decrease, but we got bigger, older preterm babies. In this form, are we doing something? We eliminated many of those that weighed <750 gm, <1000 gm, and <1250 gm. Our 10% low-birth-weight neonates are bigger preterm babies and slightly older. Maybe we are doing something.

Dr. Kirschbaum. Are there any other outcome measurements? Are there fewer neonatal intensive care unit admissions and shorter times of respiratory assist?

Dr. Harbert. Yes, as you would expect with a larger baby, a 2000 gm baby versus a 1500 gm baby. We are getting bigger and slightly older preterm babies.

Dr. Kirschbaum. You're either doing something or you're forcing immigration to other states.

Dr. Harbert. I just want to know if Dr. Pauerstein and the people on the panel have looked at these things. Especially Dr. Creasy, have you looked at this? Are we getting a little further along?

Dr. Pauerstein. We did look at it in the analysis, and we were unable to show any impact beyond the very narrow criteria that I gave you. When you look at the analysis, you can't find any improvement in terms of all the other things that were asked, all the other variables mentioned; so yes, we did look at it. I think the State of Virginia is to be admired for this, and I would like to learn more about how you do it.

Dr. Harbert. Maybe it's a fluke of our beautiful climate here.

Dr. Creasy. I don't think there's any question that there have been methods that appear to have worked in different settings and in different countries

throughout the world. In the Papiernik experience, a decrease occurred in Haguenau; then when they sampled the whole country, they had a significant decrease. A couple of important issues, however.

One is it took 3 years before any significant decrease occurred, when there was really a social intervention; and then, as Breart, one of his colleagues, reported in the 1980s, the incidence of preterm birth went back up, supposedly because there was something different in the social report mechanisms that were being used by the new government.

There certainly are programs that have worked. We did a study in northern California. The whole issue, to go back to what Dr. Nelson said earlier, was that in the second year of that study Medicaid support suddenly disappeared. So we had an increasing incidence of premature birth with that occurring.

Despite that, there was roughly a 20% reduction in premature birth. Some things do work, but my summary was to try to put things into perspective. From a national basis we have not decreased the incidence of premature birth.

Dr. Kirschbaum. We can't let this go without saying what Dr. Charles Hendricks said long ago: The way to abolish preterm labor is to abolish poverty.

Dr. Irwin R. Merkatz, Bronx, New York. I would like to pick up on Dr. Creasy's final comment acknowledging that we have yet to demonstrate a measurable impact on the overall incidence of preterm birth in this country. Nevertheless, prematurity remains the number one problem in obstetrics, and as in so many other public policy issues, not to decide our discipline's position with respect to tocolytic therapies would indeed be paramount to deciding. The opinions we have heard expressed earlier as to whether in this university service we're going to use a specific agent or in that university service we're not going to use that drug put our specialty in very delicate position. It makes us vulnerable to have such a critical decision made instead by journalists, by insurance companies, by lawyers, or by health policy makers external to the profession. I believe therefore that it is most critical that American obstetricians come to a consensus position about the use of tocolytic therapy and its limitations, even while we advocate for expanded clinical research in this important area.

There are very few physicians responsible for running busy obstetric services who cannot point to multiple instances where intervention with tocolytic therapy has made a major difference in the outcome of individual pregnancies. There exists a huge discrepancy between day-to-day experience in caring for patients and the available epidemiologic analyses. A great deal of this discrepancy is due to the fact that these drugs have often been used excessively and at times indiscriminately, to a large extent for the wrong patients. When a large number of "wrong" patients are included in the studies, that dilutes any potential opportunity to demonstrate the true

benefits for the selected few for whom the agents are truly indicated.

Dr. Creasy, in a comment while addressing another questin, said that, as far as he was concerned, he was not at this point in a position to abolish the use of tocolytic therapy on his service. I would strongly second that opinion, and I think it important to get a feeling from the leadership of our profession as to where the majority of us stand in that regard and to make that conclusion clear to the public at large.

We have had allusions today two or three times to the Canadian multicenter trial of ritodine for tocolysis (see reference 24 of article by Higby et al). It was The New England Journal of Medicine publication of that Canadian study along with an accompanying inflammatory editorial that triggered expose-like coverage by The New York Times. In turn, this led to wide national and international coverage with a lot of one-sided front page newspaper attention all across the United States. If that is how we are going to permit our obstetric policy decisions about the care of high-risk mothers and babies to be made, I think we're in for big troubles as a specialty in the future.

Dr. Kirschbaum. But the conflict between intuitive observations and truth is settled by controlling those observations. I think those of us who are critical of the use of tocolytics wish that someone would produce a controlled observation that demonstrates their usefulness.

Dr. James Woods. Memphis, Tennessee. An article recently came out describing the experience in Phoenix of this group that delivered quadruplets, 10 or 11 sets. Most of them were beyond 31 or 32 weeks' gestation.

I talked to some individuals who were involved in perinatal care for a group of patients with in vitro fertilizations, and one of their philosophies is to encourage these patients to gain as much weight as possible. They have a very distinct approach.

Would you comment on whether you think that that has been underemphasized in our management of these patients?

Dr. Creasy. I think the work that Dr. Barbara Abrams did at Berkeley in California has clearly demonstrated that poor nutritional status, or lack of weight gain is a feature that is correlated with premature birth. There's no question at all about that now. I used to work with her a long time ago and in good faith said to her, where are the solid data to support that thesis, because I couldn't see it. I think she has now definitely given us those data.

What has not been shown is clear evidence that nutritional supplementation, in some way, shape, or form, encouraging greater weight gain has resulted in an improvement in premature birth.

Indeed, the famous study of protein supplementation actually showed a worsening effect. That doesn't mean that caloric supplementation would not be of benefit, and I think it just needs to be tried. I'm a firm believer, but I don't have the hard data to support it.

Dr. Ronald S. Gibbs, Denver, Colorado. Dr. Kirschbaum's very fair and thorough evaluation has addressed antibiotics for the purpose of prevention of preterm birth. I just wanted to emphasize that there may be other compelling reasons for treatment of infections otherwise, such as prenatal treatment of chlamydia to prevent spread of a sexually transmitted disease and to prevent neonatal infection with chlamydia and, as another example, treatment for group B streptococci.

Dr. Kirschbaum. Dr. Gibbs is correct, of course. He has a couple of publications that I did not present to you, because they were outside of my purview, demonstrating the usefulness, from the point of view of neonatal care, of treating women with antibiotics.

His reference to the group focusing on bacteriuria is to Thomason from Denmark. That study demonstrated that the treatment of group B streptococcal bacteriuria brought a significant improvement in survival. The problem is not with the treatment group. The problem is with a control group because that control group had a 38% incidence of premature rupture of membranes and 53% incidence of prematurity. If those individuals only had bacteriuria, that's strange behavior. Those were probably women with chronic activity urinary tract infection or something else, and I think that casts some doubt on their results.

Dr. John T. Queenan, Washington, D.C. Today's presentations have unfortunately been negative, and I believe they have promoted the concept that there are no efficacious pharmacologic agents. It is true that much of the data supports the fact that many interventions are not effective for stopping premature labor, but there are studies indicating that some of these agents, such as protaglandin inhibitors and antibiotics, do work.

We must remember that, to data, we have examined few pharmacologic agents. One of the reasons for this limited research has been the pharmaceutical industry's lack of interest in creating new modalities. The development of new drugs for tocolysis carries the unwanted risk of product liability.

It takes approximately 10 years for an idea to get from the drawing board into practice. We are approaching an impasse now, but we can open our options by encouraging the participation of the Food and Drug Administration, the National Institutes of Health, and pharmaceutical industry in the search for more agents.

Dr. Creasy. Dr. Queenan, I hope you don't think I was being totally negative in my remarks. If you look at the reviews that I cited, at least three of them

done by unbiased or uninterested individuals, one of them obviously, as exemplified by letters to the editor, seems to have a particular issue at stake; but the three of them end up saying that there has been some benefit by these home uterine activity monitoring systems in some way, shape, or form that people can't really get a handle on.

So I think we should not be totally negative at this time. I was just trying to present hard information.

Dr. Queenan. I'm referring also to the pharmacologic approach to the problem.

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Prolonged Blockade of Nitric Oxide Synthesi in Gravid Rats Produces Sustained Hypertension, Proteinuria, Thrombocytopenia, and Intrauterine Growth Retardation

[Basic Science Section]

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Outline

Graphics

Abstract

OBJECTIVE: Our purpose was to test the hypothesis that chronic inhibition of nitric oxide synthesis in pregnant rats can produce a preeclampsia-like syndrome.

STUDY DESIGN: Pregnant rats were instrumented on day 14 of gestation (parturition day 21 to 22) and infused continuously through a venous catheter with L-nitro-arginine, a potent inhibitor of nitric oxide synthase, or with sterile saline solution from day 18 until 24 hours post partum. A group of virgin rats was treated identically. Blood pressure was recorded in unrestrained animals with an aortic catheter for 30 minutes before infusion and repeated each day throughout the experiment. Urinary albumin, platelet count, weight of newborn pups, blood chemistry, and several other parameters were determined. Data were analyzed by one-way, repeated-measures analysis of variance, with Dunnett's t test or by Student t test.

RESULTS: Mean arterial pressure increased from 102.6 +/-2.8 to a mean maximum of 152.5 +/-7.3 on the second day of infusion and remained in this range until delivery, after which it fell significantly, in spite of continuing infusion of L-nitro-arginine. This treatment increased urinary albumin (milligrams per 24 hours) from 8.3 +/-1.5 to 56.3 +/-14.3 in gravid and from 8.2 +/-0.8 to 18.2 +/-2.4 in virgin rats. Weight of newborn pups was reduced by L-nitro-arginine from 5.62 +/-0.10 to 3.37 +/-0.32 gm ($p < 0.005$) without affecting time of delivery or litter size. Platelet count was reduced 58% in gravid and 50% in virgin rats.

CONCLUSION: Chronic inhibition of nitric oxide synthesis in gravid rats leads to sustained hypertension, proteinuria, thrombocytopenia, and intrauterine growth retardation, providing a simple animal model for preeclampsia. (AM J OBSTET GYNECOL 1994;170:1458-66.)

Key words: Inhibition of nitric oxide, preeclampsia, L-nitro-arginine, hypertension, fetal growth retardation

There is abundant evidence in the literature that the vascular endothelium produces a number of potent vasoactive agents that act in paracrine fashion on adjacent smooth muscle cells to regulate vascular tone [1]. A delicate balance between vasoconstrictors such as endothelins and vasorelaxants (e.g., endothelium-derived relaxing factor (EDRF) and prostacyclin) may play an important role in the maintenance of normal blood pressure. Compared with the nonpregnant state the gravid rat, as does the pregnant woman, exhibits a markedly reduced responsiveness to vasopressor agents such as angiotensin II, norepinephrine, and vasopressin [2,3]. Although the reduced peripheral vascular resistance, believed to be responsible for this phenomenon, is poorly understood, vasodilator prostaglandins, elaborated chiefly by endothelial cells, have frequently been invoked as active agents. Furthermore, the reversal of this key hemodynamic parameter in pregnancy-induced hypertension has often been attributed to a reduction in the ratio of vasodilator prostanoids, particularly that of prostacyclin and the vasoconstrictor thromboxane [4]. More recent studies, however, have provided strong evidence that nitric oxide (believed to be identical with EDRF), [5] synthesized by endothelial cells from the amino acid L-arginine, [6] plays a pivotal role in the maintenance of normal vascular tone [7]. Acute blockade of nitric oxide synthesis by arginine analogs such as L-nitro-arginine methylester (L-NAME), leads to transient but marked hypertension in both gravid and nonpregnant rats [3]. Moreover, the typical refractoriness of pregnant rats to vasopressors was abolished by L-NAME

and was indistinguishable from dose responses elicited by these agents in nonpregnant animals [3]. Finally, the hypertensive effect of L-NAME could be reversed by the administration of excess L-arginine, [3] a frequently used approach to demonstrate the specificity of the effect evoked by the inhibitor. Prompted by these observations, we undertook the current study to evaluate the possibility that prolonged administration of L-NAME to chronically instrumented pregnant rats may induce a preeclampsia-like syndrome, thereby adding further support to the notion that reduced nitric oxide production by damaged endothelium plays an important role in the development of pregnancy-induced hypertension, preeclampsia, or eclampsia. While these experiments were in progress Baylis and Engels [8] reported that chronic administration of L-NAME in drinking water produced hypertension and proteinuria in pregnant rats, with relatively poor maternal and fetal outcome.

Material and methods

Reagents. Ketamine hydrochloride (Ketanest) and pentobarbital sodium (Nembutal) were purchased from Parke-Davis (Munchen, Germany) and from Veterinary Laboratories (Lenexa, Kan.), respectively. L-NAME and all other chemicals used in this study were obtained from Sigma (St. Louis).

Experimental protocol. Wistar-derived, timed-pregnant and virgin rats (LATI, Godollo, Hungary) were housed individually in standard metabolic cages, allowing for the collection of urine and recording of food and water consumption throughout the study. The guidelines approved by the animal research committee of Semmelweis Medical University for the care and use of experimental animals were closely observed. On the fourteenth day of gestation (parturition 21 to 22 days) one arterial and one venous catheter were implanted, as previously described by Fejes-Toth et al [9]

Four days later a continuous infusion of L-NAME, dissolved in sterile saline solution, was started at the rate of 0.5 mg in a volume of 0.05 ml per 100 gm of body weight per hour through the catheter implanted into the vena cava. Control pregnant animals were treated with saline solution alone at the same rate. Pups were removed and weighed immediately after delivery. Infusions were continued at the same rate for an additional day post partum. Virgin rats were instrumented and treated with L-NAME or saline solution, as described for pregnant animals. Every day, starting at 3 PM, mean arterial pressures (MAP) were recorded in conscious, freely moving rats by means of an electromanometer (EM 61, Medicor, Budapest, Hungary) and a Statham P23dB pressure transducer (Hato Rey, Puerto Rico). The experimental protocol is summarized in Figure 1.

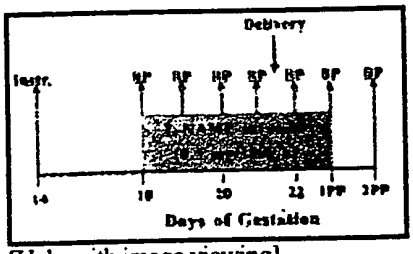

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Figure 1. Illustration of experimental protocol. Rats were instrumented under anesthesia on day 14 of gestation, and the infusion commenced on day 18, lasting until 1 day post partum. Animals were delivered between days 21 and 22. Blood pressure (BP) was recorded before, and every day during, infusion

Sample collection and analysis. Body weight, water and food intake, and urine volume were monitored daily. Twenty-four--hour urine samples were collected, centrifuged at 3000g for 15 minutes and stored at -20 degrees C until chemical analyses were performed (usually <2 weeks) for albumin, creatinine, sodium, potassium, and gamma-glutamyltransferase (EC 2.3.2.2). Blood samples were withdrawn into syringes shortly before the infusion was started (for baseline values) from the catheter implanted into the aorta and 4 days later to evaluate the effects of L-NAME on the following laboratory parameters: albumin, creatinine, sodium, potassium, gamma-glutamyltransferase, aspartate aminotransferase (EC 2.6.1.2), blood urea nitrogen, glucose, triglycerides, and cholesterol. Serum and urine sodium and potassium were measured by atomic absorption spectrophotometry (PYE UNICAM SP 191, Cambridge, U.K.). All other measurements were carried out on a Technicon RA-100 (Tarrytown, N.Y.) automatic laboratory system, using the appropriate Technicon diagnostic test reagents. Creatinine clearance and daily sodium, potassium, and albumin excretion were then calculated. Platelet count was determined according to Brecher and Cronkite [10]. Plasma volume was estimated by the Evans blue disappearance method [11]. Briefly, blood (0.2 ml) was collected through the aortic catheter. Next, Evans blue (2 mg/kg) was injected in a volume of 50 microliters through the venous catheter and washed in with 0.2 ml of saline solution. Ten minutes later blood samples were collected in triplicate with hematocrit capillaries, followed by four subsequent samplings at 10-minute intervals. The hematocrit tubes were centrifuged at 3000 revolutions/min for 20 minutes, and the optical density of the sera was read at 620 nm. The dye concentration was calculated from the standard curve. The values were plotted against time, and plasma volume was calculated from the Y intercept of the best-fit line between data points of 10 to 40 minutes obtained by least-squares regression analysis.

Renal histologic characteristics. Pregnant and virgin rats were instrumented and treated with L-NAME or saline solution, using the same infusion rate as above. After 4 days of treatment the animals were anesthetized with pentobarbital sodium (30 mg/kg), and both kidneys were removed, weighed, and the left one processed for light microscopic examination. Coronal kidney sections were fixed in 10% buffered formalin, dehydrated through ascending grades of alcohol, and embedded in paraffin. Sections (3 microns thick) were stained with hematoxylin and eosin, periodic acid--Schiff, or Golgner's trichrome and periodic acid--methenamine silver. Sections were

examined on a blinded basis for the level of glomerular damage. A minimum of 50 glomeruli in each specimen was evaluated, and the severity of the lesion was graded 0 to 4+, according to Raij et al [12]

Statistical analysis. Results are presented as mean +/-SEM. MAP changes during infusion of L-NAME were statistically analyzed by one-way repeated-measures analysis of variance and post hoc Dunnett's t test. Comparisons of corresponding values of pregnant and virgin rats were made by Student t test. Probability level of <0.05 was accepted as statistically significant.

Results

The mean body weight, food and water intake, and urine volume before (control) and on the third day of infusion (treated) are summarized in Table I. In spite of similar food intake, the body weight of L-NAME--treated mothers was lower than that of saline solution--treated controls, probably reflecting the significant growth retardation of conceptuses. Interestingly, L-NAME infusion significantly increased urine volume in both pregnant and virgin groups compared with corresponding saline solution--treated groups.

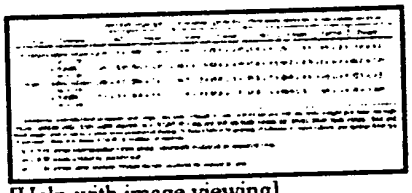

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Table I. Effect of chronic L-NAME infusion on body weight, food and water intake, and urine volume of instrumented gravid and virgin rats

The basal MAP of gravid rats on day 14 of gestation (at the time of implantation of catheters) and on day 18 (just before the start of infusion) were significantly lower (102.6 +/-2.8 mm Hg) than in virgin rats at the corresponding times (113.5 +/-3.5 mm Hg). Within 24 hours MAP in the pregnant group increased to 132.6 +/-4.5 mm Hg ($p < 0.001$), reaching mean maximum values of 152.5 +/-7.3 mm Hg 2 days after the onset of continuous L-NAME infusion. This marked hypertension was sustained until delivery at term, after which blood pressure fell rapidly, in spite of the continuing administration of L-NAME at the same rate for an additional day post partum. However, MAP still remained significantly above preinfusion levels. In contrast, MAP in the virgin group rose more moderately, from 113.5 +/-7.05 mm Hg to 134.9 +/-3.8 mm Hg by 24 hours after the start of infusion, followed by a progressive decline to a level that was sustained above baseline for the rest of the experimental period Figure 2.

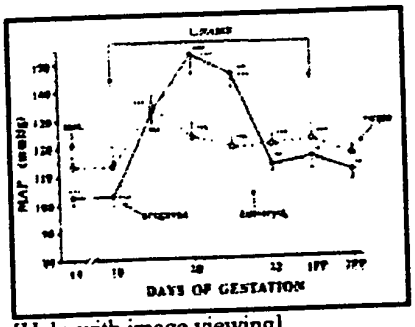

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Figure 2. Effect of chronic L-NAME infusion (0.5 mg/100 gm/hr) on MAP of instrumented pregnant (filled circles. n = 12) and virgin (open circles, n = 14) rats. Two asterisks, $p < 0.01$; three asterisks. $p < 0.005$ versus preinfusion values by one-way repeated analysis of variance and post hoc Dunnett's test; three daggers, $p < 0.005$ versus virgin animals by unpaired t test

Infusion of L-NAME from day 18 of gestation caused a significant retardation of fetal growth compared with controls receiving saline solution alone (5.62 +/-0.10 gm vs 3.37 +/-0.32 gm, $p < 0.001$) without affecting litter size or the duration of gestation. Whether intrauterine growth retardation (IUGR) was associated with smaller than normal placentas remains uncertain because the mothers cannibalized many of them before they could be retrieved for weighing. Furthermore, the mean daily excretion of urinary albumin was increased dramatically by L-NAME in gravid rats, from (in milligrams per 24 hours) 8.3 +/-1.5 to 56.3 +/-14.3 ($p < 0.005$) and from 8.2 +/-0.8 to 18.2 +/-2.4 ($p < 0.005$) in the virgin group.

Glomerular filtration rate, as estimated by creatinine clearance, which was significantly elevated in pregnant versus virgin rats, was markedly reduced by L-NAME infusion in the gravid but not in the nonpregnant animals Table II. At the same time urinary gamma-glutamyltransferase excretion, a useful index of nephrotoxicity in rats, [13] increased strikingly in both groups, although the increase was more than twice as much in the pregnant group Table II. Similarly, chronic blockade of nitric oxide synthase caused a significant increase in urinary sodium excretion in both gravid and virgin rats, without affecting potassium excretion Table III. We have also observed a significant decrease in platelet count as a result of L-NAME infusion and a modest but statistically significant increase in hematocrit in the pregnant group compared with corresponding saline solution--infused rats Table IV.

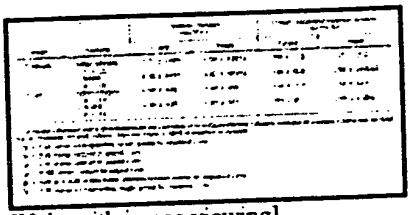

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Table II. Effects of L-NAME treatment on creatinine clearance and gamma-glutamyltransferase excretion rates in chronically instrumented gravid and virgin rats

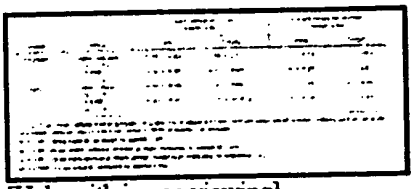

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Table III. Effects of chronic infusion of L-NAME on urinary sodium and potassium excretion in gravid and virgin rats

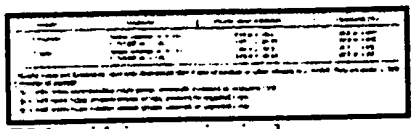

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Table IV. Effect of infusion of L-NAME on platelet count and hematocrit values in chronically instrumented gravid and virgin rats

With the exception of triglycerides, which were reduced in pregnant rats from 2.44 +/-0.28 mmol/L (saline solution infusion) to 1.35 +/-0.16 mmol/L (L-NAME infusion, $p < 0.05$), and in the corresponding treatments of virgin rats from 1.13 +/-0.13 mmol/L to 0.81 +/-0.08 mmol/L ($p < 0.05$) L-NAME treatment had no distinguishable effect on any of the other serum laboratory values compared with specimens collected from saline solution--treated controls (results not shown).

The rate of disappearance of Evans blue from the circulation was fastest in gravid animals treated with L-NAME, which also accelerated, although to a lesser extent, the clearance of this albumin-bound dye in the nonpregnant group Figure 3. Plasma volume, calculated from data of the dye experiment, demonstrated that the pregnancy-induced expansion observed in saline solution--treated rats was reduced to near nonpregnant levels by chronic blockade of nitric oxide synthase Figure 4. Administration of L-NAME was associated with severe glomerular morphologic alterations in pregnant animals Figure 5. Glomerular capillary lumens were segmentally occluded by intraluminal masses of eosinophilic material. This intracapillary substance stained fuchsinophilic with trichrome stain and partially positive with fibrin stains. Extraglomerular lumens were filled with protein cylinders. In addition, a mild diffuse interstitial edema and a sparse interstitial infiltrate of lymphocytes were observed. L-NAME treatment did not bring about these changes in the kidneys of virgin rats during the 4-day experimental period. A semiquantitative histologic evaluation of renal injury using a 0 to 4+ scale is presented in Table V.

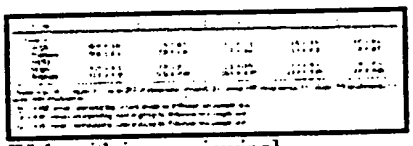

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Table V. Glomerular injury score of controls and L-NAME--treated rats

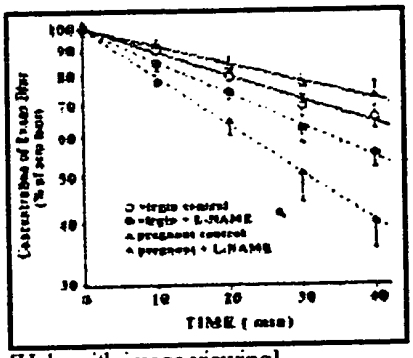

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Figure 3. Effect of chronic L-NAME treatment on Evans blue elimination in pregnant and virgin rats. Dye disappearance was determined in same animals before (control) and 3 days after start of infusion (closed symbols). L-NAME–treated animals significantly ($p < 0.05$) different from control values. Pregnant values $p < 0.01$ versus virgins

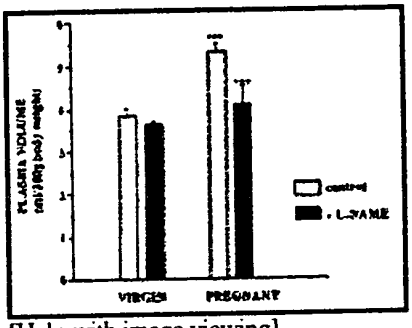

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Figure 4. Effect of L-NAME treatment on plasma volume as estimated from Evans blue elimination data Figure 3

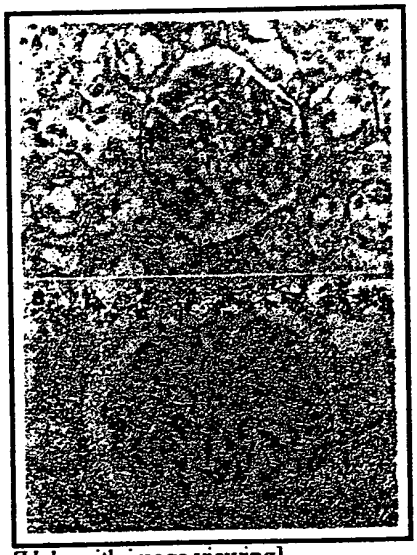

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Figure 5. Representative photomicrographs of glomeruli from virgin (A) and pregnant (B) rats given L-NAME infusion for 4 days. Kidneys were removed and examined under light microscopy with hematoxylin and eosin staining. Glomeruli from pregnant, but not virgin, animals showed typical signs of focal segmental glomerulosclerosis

Comment

A previous study [3] has demonstrated that an 8-hour infusion of L-NAME brought about a marked hypertension in chronically instrumented conscious rats and abolished the refractoriness to angiotensin II, vasopressin, and norepinephrine, a hallmark of hemodynamic changes surrounding preeclampsia [14,15]. By means of the same animal model the current

investigation was undertaken to evaluate the long-term effects of controlled, continuous intravenous infusion of L-NAME on blood pressure, fetal development, platelet count, and some other parameters in both gravid and virgin rats. The results of this study have demonstrated that chronic inhibition of nitric oxide synthesis during late pregnancy produces sustained hypertension that subsides after parturition. In nonpregnant virgin rats MAP, after a significant initial rise during the first 24 hours, progressively declined, remaining nevertheless significantly above preinfusion basal levels. In male rats chronic administration of L-NAME offered in the drinking water for 2 months produced a slowly evolving but sustained hypertension and proteinuria [16]. Similarly, administration of L-NAME by gavage twice a day for 4 weeks produced a dose-dependent increase in blood pressure [17]. Taken together, these and some other recent studies [18] demonstrate that chronic inhibition of nitric oxide synthesis alone is sufficient to induce hypertension, supporting a critical role attributed to nitric oxide in the regulation of vascular tone. However, the results of the current investigation have shown that pregnant rats are particularly prone to sustained hypertension and its sequelae, in response to chronic inhibition of nitric oxide synthesis. The obvious implication of this observation is that the fetoplacental unit is a major source of nitric oxide, fulfilling a crucial role in blood pressure regulation during pregnancy. Indeed, a recent study by Conrad et al [19] presented convincing evidence that nitric oxide production increases during pregnancy in rats. Such a mechanism is concordant with the belief that the fetoplacental unit is a major component of the mechanism underlying the pathophysiologic mechanisms of preeclampsia [20]. The return to normotension after parturition of patients with pregnancy-induced hypertension or preeclampsia is analogous to the fall of blood pressure in the current rat model within a few hours after the delivery of the pups and placentas.

Moreover, the finding of this study that arterial pressure declined precipitately after parturition, in spite of continuing infusion of the nitric oxide synthase inhibitor even at a slightly higher rate relative to body weight, supports the notion that the poorly perfused preeclamptic placenta elaborates a substance, or substances, that exacerbate the syndrome [15]. It would appear that, once the pregnant uterus is evacuated, the consequences of nitric oxide synthesis blockade are less severe and may resemble those of nonpregnant rats maintained under the same experimental regimen. This interpretation is consistent with our observations that almost all other parameters affected by nitric oxide blockade were markedly amplified in gravid animals.

The second important finding of this study was the significant IUGR in L-NAME--treated rats. This phenomenon, which frequently accompanies preeclampsia, has been attributed to inadequate fetoplacental perfusion, [20] because it can be readily obtained by surgical restriction of uterine blood supply in experimental animals [21,22]. Clearly the inhibition of nitric oxide synthesis by the chronic infusion of L-NAME is sufficient to produce IUGR, adding further support to the role this endothelial factor plays in the

regulation of normal vascular function in pregnancy. It is of note that, besides IUGR, no ill effects were observed in L-NAME--treated rats, all of which were delivered of live pups at term. In contrast, administration of L-NAME in the drinking water starting at 3 to 4 days of pregnancy produced deleterious effects, resulting in occasional maternal and fetal deaths [8]. This discrepancy may have been related to the early and more prolonged exposure of gravid rats to L-NAME in the study of Baylis and Engels [8] compared with the protocol used in this study.

We made no attempt in this study to reverse the effects of L-NAME with the administration of the nitric oxide precursor L-arginine, because previous experiments [3] have convincingly shown that such intervention can effectively inhibit the action of L-NAME on blood pressure and heart rate in pregnant and nonpregnant rats.

The third significant finding of this study is the marked proteinuria observed in both virgin, and particularly pregnant, L-NAME--treated rats. This is consistent with recent findings of other investigators using both male and female rats [8,16] and signifies impaired glomerular permeability, one of the diagnostic triads of preeclampsia. Glomerular capillary hypertension or mesangial hypertrophy, leading to glomerular sclerotic injury, may be responsible for such glomerular damage in response to chronic inhibition of nitric oxide synthesis [16]. The results of this study are concordant with these observations, clearly showing marked structural alterations in glomeruli of L-NAME--treated pregnant rats. Of particular interest is the finding that, under the current experimental conditions, the same treatment of virgin females did not elicit such glomerular lesions, pointing once again to the exquisite sensitivity and reliance of gravid animals on endogenous production of nitric oxide. However, an increased rate of Evans blue disappearance points to a more generalized endothelial permeability. Here again chronic L-NAME infusion provoked a markedly more pronounced effect in pregnant animals. These observations are consistent with those reported for preeclamptic humans [23] and support the concept of endothelial injury as the underlying pathogenetic cause of this syndrome [24].

Of particular interest is the marked decrease in platelet count in both pregnant and virgin L-NAME--treated rats. Thrombocytopenia is frequently associated with preeclampsia, [25] from enhanced aggregability, and adhesion to damaged endothelium. It is known that nitric oxide inhibits both aggregation and platelet adhesion to endothelial cells [26]. Therefore the observed decrease in platelet count in chronically nitric oxide--blocked animals may reflect one or both of these actions of this endogenous vasodilator.

The mechanisms of L-NAME--induced diuresis is unknown. It has been reported recently that infusion of 30 gm of L-arginine to normal human subjects produced hypotension and significant diuresis [27]. These investigators suggested that it might have resulted from increased renal

plasma flow, because of stimulation of endogenous EDRF--nitric oxide formation from L-arginine. However, in view of L-NAME--induced diuresis in the current study it would appear that the amino acid analog itself is diuretic and that this is unrelated to nitric oxide blockade. Indeed, a recent study [28] has demonstrated that, in addition to L-arginine, several other amino acids that cannot serve as nitric oxide donors also possessed diuretic and natriuretic activities in conscious, unrestrained Wistar rats. However, a more recent study [29] presented evidence that nitric oxide, acting centrally as a neurotransmitter or neuromodulator, stimulated antidiuretic hormone release in rats. Such a mechanism may have accounted for the observed diuresis in both virgin and nitric oxide--blocked pregnant animals. Indeed, preliminary results in our laboratories, showing a significant decrease in the specific gravity of urine from L-NAME--treated rats, support this notion.

In conclusion, we have presented evidence that inhibition of nitric oxide synthesis during the latter part of pregnancy by continuous infusion of L-NAME to chronically instrumented, conscious, freely moving rats produced a preeclampsia-like syndrome that was characterized by hypertension, proteinuria, thrombocytopenia, IUGR, and marked glomerular damage, all known corollaries of the human disease. This approach may offer a well-controlled, useful model to study this serious disorder of human gestation. The results, although implicitly, also indicate that continuous generation of EDRF--nitric oxide is essential for the maintenance of normal gestation. Although an alteration in the relative production of prostacyclin and thromboxane has received the most attention as a possible causal factor in the development of preeclampsia, there is no convincing evidence that, in an experimental animal model, the inhibition of or the alteration of the ratio of these vasoactive agents can bring about a syndrome akin to preeclampsia. It seems more likely therefore that these prostanoids play a secondary, complementary role in the maintenance of blood supply to the fetoplacental unit, which depends critically on the endothelial production of EDRF--nitric oxide.

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Fetus-Placenta-Newborn: Nitric Oxide Inhibition Causes Intrauterine Growth Retardation and Hind-Limb Disruptions in Rats

[General Obstetrics And Gynecology]

Diket, Albert L.; Pierce, Maria R.; Munshi, Upender K.; Voelker, Cynthia A.; Eloby-Childress, Sandra; Greenberg, Stanley S.; Zhang, Xiao-Jing; Clark, David A.; Miller, Mark J. S.

Outline

Graphics

Abstract

OBJECTIVE: Our purpose was to determine the effects of nitric oxide synthase inhibition on maternal and fetal health in the last third of pregnancy.

STUDY DESIGN: Pregnant rats were treated from gestational day 13 to day 19 or 20 with the nitric oxide synthase inhibitor N^G-nitro-L-arginine methyl ester, which was administered in the drinking water ad libitum. Control animals received the inactive enantiomer N^G-nitro-D-arginine methyl ester or no treatment. Maternal blood pressure, blood chemistry studies, and placenta and pup size were determined. A separate group of rats received nitroprusside sodium in conjunction with N^G-nitro-L-arginine methyl ester.

RESULTS: N^G -nitro-L-arginine methyl ester caused a dose-dependent reduction in placenta and pup size. Amniotic fluid levels of cyclic guanosine monophosphate were significantly reduced at 0.1 mg/ml but not at higher doses. Hemorrhagic necrosis of fetal hind limbs occurred only with treatment with N^G -nitro-L-arginine methyl ester and was prevented by coadministration of nitroprusside sodium. Maternal blood pressure and blood and urin chemistry studies were unaffected by N^G -nitro-L-arginine methyl ester.

CONCLUSION: Chronic reductions of nitric oxide production in the last third of pregnancy result in significant intrauterine growth retardation and hemorrhagic disruptions of hind limbs. Maternal complications were minimal and did not mimic preeclampsia. (AM J OBSTET GYNECOL 1994;171:1243-50.)

Key words: Nitric oxide, fetus, pregnancy, rats, birth defects

Nitric oxide formation is up-regulated in pregnancy, as determined by the urinary excretion of cyclic guanosine monophosphate (cGMP) and nitrite-nitrate, [1,2] but the role of nitric oxide in maternal adjustments to pregnancy or fetal growth and development is unknown. Because it is an endothelium-derived vasorelaxant with important effects on neutrophils, platelet aggregation, and vascular tone, [3] it is possible that it contributes to the decreased maternal vascular responsiveness that accompanies normal pregnancy [4]. Nitric oxide may also be a key regulator of placental blood flow and oxygen and nutrient exchange, playing an important role in assuring fetal well-being [5,6]. Endothelial release of nitric oxide is influenced by gender. Estrogens appear to promote nitric oxide production, [7] suggesting that the altered hormonal milieu associated with pregnancy may affect nitric oxide --dependent mechanisms.

Diminished nitric oxide release is evident in experimental and clinical hypertension [8,9]. Furthermore, long-term administration of nitric oxide synthase inhibitors results in hypertension and renal impairment [10] that is reversed by an excess of the nitric oxide precursor L-arginine [11]. Preeclampsia is a major cause of maternal death and morbidity in pregnancy. Although its cause is unknown, its characteristics include hypertension, enhanced vascular reactivity, altered synthesis of endothelium-derived vasoactive mediators (prostaglandins, thromboxane, and endothelin), altered blood rheologic features, and renal and hepatic impairment [12]. Fetal effects are generally less severe and usually include growth retardation secondary to compromise of the placental circulation. Release of nitric oxide from umbilical vessels has been reported to be impaired in pregnancy-induced hypertension [13].

On the basis of the characteristics of preeclampsia, we hypothesized that inadequate formation of nitric oxide may contribute to the hypertension and end-organ dysfunction. To address this possibility, we administered the nitric oxide synthase inhibitor N^G -nitro-L-arginine methyl ester (L-NAME) in the drinking water of pregnant rats from gestational day 13 through days 19 to 20. The goal was to determine the importance of nitric oxide on

maternal health and fetal growth and development in the last trimester of pregnancy.

Methods

Treatment groups. Timed-pregnant rats (Holtzman, Harlan Sprague-Dawley, Indianapolis) were obtained on gestational day 12. After a day's acclimation rats were randomly assigned to three treatment groups; they first received the nitric oxide synthase inhibitor L-NAME in the drinking water at 0.1, 0.3, or 1 mg/ml, which approximates to 6 to 60 mg/day. The second group received the inactive enantiomer N^G-nitro-L-arginine methyl ester (D-NAME); administered at the same concentrations as L-NAME. The final group was a water-only control. Each shipment was divided into these three groups. Water containers with or without arginine analog were replenished daily. This route of nitric oxide synthase inhibitor administration has been routinely used by us [14] and others, [10] and it is a simple and reliable approach to the chronic reduction of nitric oxide synthesis in vivo. Animals were treated for 6 to 7 days (gestational days 13 to 19 or 13 to 20). In a subgroup of animals treated with L-NAME (1.0 mg/ml) the nitric oxide donor sodium nitroprusside was concurrently administered subcutaneously by implantation of an Alzet osmotic minipump (Alza, Palo Alto, Calif.) during ketamine anesthesia on day 13 of gestation at a dose of 1.0, 3.0, or 10 micrograms/kg/min. On day 20 to 21 of gestation dams were anesthetized for collection of maternal and fetal tissues and fluids.

Maternal blood pressure was recorded from a carotid cannula with a Gould pressure transducer (Statham, Cleveland) and recorded on a model 3400 Gould polygraph (Statham). Arterial blood pressure in anesthetized rats was measured continuously for 15 minutes to ensure that a stable reading was recorded. In a subgroup of animals on day 13 and 21 of gestation systolic blood pressure was measured with a pneumatic tail-cuff device (Narco-BioSystems, Houston) in animals that had been placed on a heating blanket to maintain a temperature of approximately 30 degrees C. Values were obtained from the average of four consecutive measurements. Rats were killed by anesthesia overdose after blood and bladder urine were removed for biochemical assessment. Maternal brain, lung, and liver were removed for estimation of edema (wet/dry weights). Tissue specimens were also obtained for routine histoligic studies (formalin fixation and paraffin embedding). Pups were gently removed from the uterus, and amniotic fluid was collected from each litter by pooling the fluid obtained from each pup. Pup number, pup weight, and placental weight were recorded along with the incidence of deformities and stillbirths.

All treatment and surgery protocols were approved by the institutional animal care and use committee of Louisiana State University Medical Center in New Orleans in accordance with the Declaration of Helsinki and National Institutes of Health guidelines. Rats were housed in a American Association for Accreditation of Laboratory Animal Care --accredited facility.

Blood and urine analysis. Blood analysis included leukocyte numbers, fibrinogen and fibronectin levels, and prothrombin and partial thromboplastin times performed in a commercial clinical laboratory. In some animals blood analysis was extended to include aspartate aminotransferase, alanine aminotransferase, total bilirubin, serum creatinine, lactate dehydrogenase, and platelet count. Urinalysis included protein and creatinine concentrations, as previously described [15].

Assay for cGMP. Amniotic fluid samples were acetylated before assay for cGMP with a commercial enzyme-linked immunosorbent assay kit (Oxford Biochemical Research, Oxford, Mich.). The use of this marker of nitric oxide release or activity after chronic oral administration of L-NAME and details of the assay procedure have recently been described [16].

Results

Maternal effects. In spite of administration of large doses of L-NAME, maternal blood pressure was comparable to water and D-NAME controls by either carotid artery cannulation Table I or by tail-cuff sphygmomanometry (difference between day 13 and day 21 (7 days) of L-NAME and water treatment was -2 +/-3 mm Hg and 4 +/-2 mm Hg, respectively; n = 3 rats per group). This was apparent for all doses. Maternal blood chemistry was also evaluated with 6- and 7-day treatment regimens. At the 0.1, 0.3, and 1.0 mg/ml doses of L-NAME we determined the maternal total leukocyte count, hematocrit, hemoglobin concentration, prothrombin time, partial prothromboplastin time, and fibrinogen level. Each evaluation was comparable in all three treatment groups (i.e., on the basis of these tests there was no evidence of maternal clotting complications, anemia, infection, or inflammation) (data not shown). In addition, we measured, but not at all doses, aspartate aminotransferase, alanine aminotransferase, total bilirubin, serum creatinine, blood urea nitrogen, lactate dehydrogenase, and platelet count, all of which were in the normal range for each group Table II. In addition, urine protein was evaluated at all doses of L-NAME Table II; values obtained in all three treatment groups were comparable.

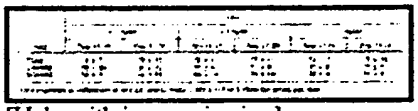

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Table I. Maternal mean arterial blood pressure

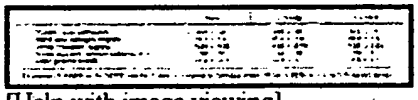

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Table II. Effect of L-NAME on index values of maternal preeclampsia

As an index of edema, we measured the wet and dry weight ratios in maternal brain and lungs and in the placenta. No evidence of edema was noted in L-NAME--treated rats. The histologic features of the kidney, liver, and placenta were comparable between the L-NAME and water treatment groups. Maternal weight gain and food consumption was comparable in each group, averaging approximately 14.8 and 35 gm per day, respectively. The presence of the nitric oxide synthase inhibitor L-NAME did not influence drinking behavior, because fluid consumption was comparable in each group, approximately 60 ml/day.

Fetal effects. Fetal growth was significantly retarded by L-NAME administration, which was evident with 6-day treatment until gestational day 19 Figure 1 or 7-day treatment until gestational day 20 Figure 2. Fetal growth retardation observed with L-NAME was dose dependent. The 0.1 mg/ml dose appeared to be the threshold dose for fetal complications, with significant effects in the 6- but not the 7-day treatment group. Marked reductions in fetal growth were observed at 0.3 and 1 mg/ml with both 6- and 7-day protocols. There was no difference in the number of pups or stillbirths in any treatment group.

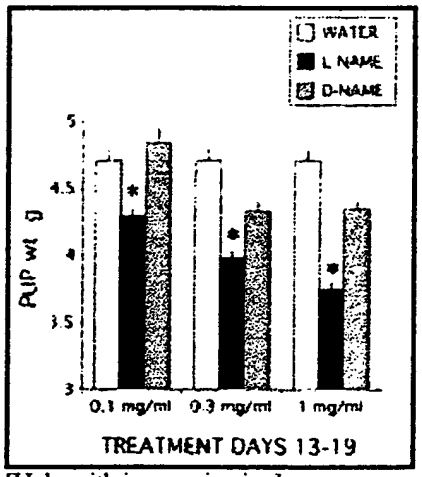

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Figure 1. Pup weights from untreated dams (control, water only) or dams treated with L-NAME or D-NAME from gestational days 13 to 19 at 0.1, 0.3, or 1.0 mg/ml in drinking water. Columns represent mean +/-SEM. Asterisk, Significant difference in L-NAME group compared with water or D-NAME controls ($p < 0.05$); $n = 57$ to 111 pups per group per dose

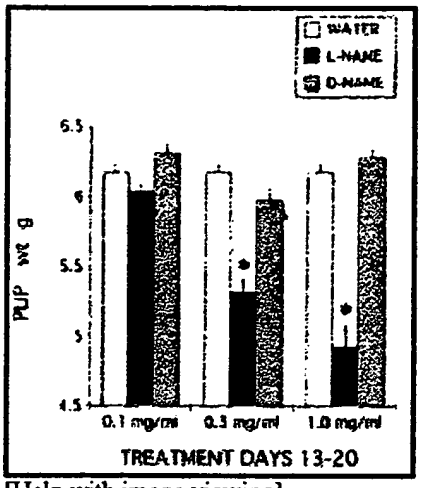

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Figure 2. Pup weights from control dams or dams treated with L-NAME or D-NAME from gestational days 13 to 20 at 0.1, 0.3, or 1.0 ng/ml in drinking water. Columns represent mean +/-SEM. Asterisk, Significant difference in L-NAME group compared with water or D-NAME controls ($p < 0.05$); n = 54 to 116 pups per group per dose

Consistent with the reductions in pup size was a dose-dependent reduction in placental weight (Figs. 3 and 4). In a manner analogous to pup size 0.1 mg/ml L-NAME was the threshold dose, reducing placental size in the 6- but not the 7-day treatment group. Marked reductions in placental size were noted with the higher doses (Figs. 3 and 4).

Amniotic fluid levels of cGMP were used as an index of fetal nitric oxide production levels of cGMP. Only the 0.1 mg/ml dose of L-NAME reduced cGMP levels Table III; at higher doses of L-NAME, cGMP values were comparable to controls.

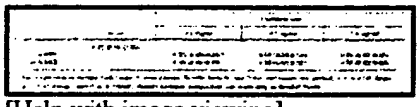

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Table III. Levels of cGMP (nanograms per milliliter) in amniotic fluid

Hind-limb disruptions in the pups of L-NAME --treated dams were a consistent finding Table IV. Disruptions were found only in the hind limbs and were observed in either leg or in both legs Figure 5. No disruptions were observed at the low dose of L-NAME (0.1 mg/ml), but disruptions were readily noted at higher doses. The incidence of disruptions was not only dose dependent, rising to 53% for the 1.0 mg/ml dose, but also influenced by the duration of exposure ($p < 0.05$). Increasing the treatment time from 6 to 7 days to include gestational day 20 as a treatment day markedly increased the incidence of these disruptions at the 0.3 and 1.0 mg/ml doses Table IV. The addition of the nitric oxide donor nitroprusside sodium to the dams receiving 1.0 mg/ml of L-NAME for 7 prenatal days reduced the incidence of disruptions in a dose-dependent manner ($p < 0.05$) Figure 6.

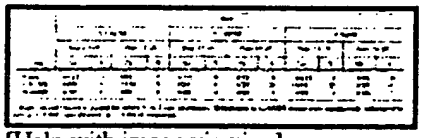

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Table IV. Incidence of fetal hind-limb deformities

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Figure 5. Gross morphologic features of a L-NAME –treated pup (left), showing bilateral hind-limb necrosis and growth retardation, compared with D-NAME control pup (right)

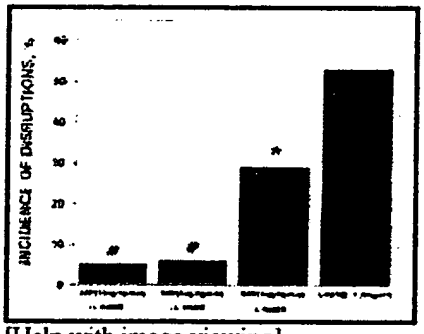

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Figure 6. Incidence of disruptions after subcutaneous administration of nitroprusside sodium (SNP) at 10, 3, and 1 micrograms/kg per minute to dams treated with L-NAME on gestational days 13 to 20. Asterisk and pound sign, Significant reversal of disruptions with addition of nitroprusside sodium with $p < 0.05$ and $p < 0.001$, respectively; n = 19 to 38 pups per group per dose

Histologic assessment of the hind limbs revealed a hemorrhagic necrosis of the hind limbs with a marked cellular infiltration and loss of structure. It is worth noting that no hind-limb disruptions were found in the 442 control pups that were evaluated in this study.

Comment

The most significant findings of this study are that administration of the nitric oxide synthase inhibitor L-NAME in the last trimester of pregnancy in

rats results in marked growth retardation and hemorrhagic necrosis of the hind limbs. These findings appear to be related to the ability of L-NAME to inhibit nitric oxide formation, because rats treated with the inactive enantiomer D-NAME were comparable to controls receiving water only. These findings with L-NAME were clearly dose dependent and, in the case of hind-limb disruptions, progressive, because the incidence of defects doubled with an additional day of treatment. Reversal of L-NAME –induced hind-limb disruptions with the nitric oxide donor sodium nitroprusside strongly suggests that these effects of L-NAME resulted from its ability to inhibit nitric oxide synthase.

Fetal growth retardation was most likely related to a compromised placental circulation, [17] limiting the maternal-fetal exchange of nutrients and metabolic wastes. Nitric oxide is an important vasodilator, and L-NAME may increase placental vascular tone [5,6]. To the best of our knowledge, all previous animal models of intrauterine growth retardation involved direct interventions on uterine blood flow or compromising maternal nutritional status or health [17,18,19]. Thus this approach represents a novel form of intrauterine growth retardation based on the inhibition of a single mediator, nitric oxide. Because maternal health was not compromised, it is suggested that adequate nitric oxide production is essential for fetal growth and development in rats. It is possible that deficits in fetal or placental nitric oxide production may contribute to clinical intrauterine growth retardation.

The ability of L-NAME to influence vascular tone in fetal vascular beds other than the placenta [5,6] is largely unknown, although nitric oxide does influence the tone of the fetal pulmonary circulation [20]. The hemorrhagic necrosis of fetal hind limbs with oral maternal L-NAME treatment suggests that L-NAME crosses the placental barrier and affects fetal nitric oxide synthesis. The dose range used in this study encompasses doses known to reduce the elevated nitric oxide production associated with experimental inflammatory bowel disease [14] and with a number of reports evaluating hypertension induced by chronic reductions of nitric oxide synthesis [10]. Thus it is likely that the results reflect an alteration of function and not chemical toxicity, particularly because there were no maternal complications and the D-NAME--treated mothers and pups were completely unaffected.

It is also possible that the effects of L-NAME may also reflect inhibition of L-arginine uptake. Because this nitric oxide synthase inhibitor is an L-arginine analog, it may compromise intracellular uptake of L-arginine, which may in turn adversely affect growth. Arginine is a semiessential amino acid. In adults dietary sources are not normally required, but in growth states and during infection or immune activation endogenous arginine production is insufficient to match demands for growth and host defense. Thus the effects of L-NAME may reflect a reduction in L-arginine bioavailability and an inhibition of nitric oxide formation.

Amniotic fluid levels of cGMP were evaluated but did not display a dose-dependent reduction with L-NAME. Although nitric oxide stimulates

soluble guanylate cyclase activity, this enzyme may be influenced by other mediators involved in pregnancy, or, alternatively, cGMP levels may be determined by activators of particulate guanylate cyclase (e.g., atrial natriuretic peptide).

A working hypothesis of this study was that chronic reductions in nitric oxide synthesis in pregnancy would establish a state similar to preeclampsia. This hypothesis was based on the proteinuria, hypertension, and enhanced responsiveness to vasoconstrictors evident in preeclampsia, which is seen with nitric oxide synthase inhibition in nonpregnant animals. In addition, endothelium-dependent vasorelaxation in umbilical vessels is impaired in preeclampsia, [13] and maternal plasma levels of the endogenous nitric oxide synthase inhibitor N^G,N^G dimethylarginine is increased in preeclampsia [21].

Nevertheless, the current study failed to mimic preeclampsia. L-NAME treatment did not affect any index of maternal health in spite of the marked effects on the fetus. Our results differ from those of Yallampalli and Garfield, [22] who reported, in addition to fetal growth retardation, an increase in maternal systolic blood pressure (by tail cuff) and proteinuria after prenatal L-NAME was administered (25 to 50 mg/day) continuously by subcutaneous osmotic pumps beginning on day 17 of gestation. In addition to proteinuria, we examined a multitude of variables that can be associated with preeclampsia. None of these were affected by L-NAME treatment, further supporting the lack of effect on blood pressure. These differences cannot be explained by mode of administration or dose. The doses of L-NAME used in our study (6 to 60 mg/day) encompass the dose range used by Yallampalli and Garfield [22]. The contrasting routes of administration (oral versus subcutaneous) still resulted in fetal growth retardation. In addition, the hind-limb disruptions observed in our study strongly suggest that L-NAME was given in high enough concentrations to cross the maternal gut and placental barriers.

The lack of hypertensive effects with L-NAME in our study cannot be explained by mode of maternal blood pressure measurement, because carotid artery cannulation is considered a more reliable and accurate technique than tail-cuff sphygmomanometry (our tail cuff subgroup also did not reveal differences between pretreatment and 7 days of treatment). On the basis of our results, we are forced to conclude that maternal exposure to L-NAME does not establish a model of preeclampsia. It is possible that the maternal capacity to generate nitric oxide in pregnancy is dramatically up-regulated to an extent that it is resistant to complete inhibition.

Unlike the mother, the fetus appears to be sensitive to vasoconstrictor effects of L-NAME. The hind-limb disruptions, which originate at the extremities (toes, footplates, and tibia) appear grossly and histologically similar to hemorrhagic necrosis. Peripheral extremities are susceptible to vascular compromise leading to necrosis and absorption, as has been described with cocaine exposure, [23] caffeine-induced catecholamine

surges, [24] and possibly bleeding resulting from chorionic villus sampling [25]. These agents cause limb or extremity disruptions in the late organogenic or postorganogenic period, further supporting a vascular dysfunction. This is further strongly supported by significant reversal of disruptions after addition of the nitric oxide donor nitroprusside sodium to L-NAME prenatal treatment. The teratogenic effects of L-NAME are likely to be post- organogenic. L-NAME was first introduced on day 13, and the incidence of disruptions was significantly influenced by the duration of exposure, doubling with an additional day of treatment (day 20). Thus the pathophysiologic process was still occurring close to term (day 22). L-NAME is an inhibitor of nitric oxide synthase, with a slight selectivity for the constitutive (neuronal, endothelial) over the inducible (immune) forms. It is unknown whether inhibitors that are selective for the inducible form of nitric oxide synthase, which should not compromise vascular function, are teratogenic. The location and nature of the hind-limb defects are suggestive that inhibition of the constitutive (vascular) form of nitric oxide synthase is responsible for growth retardation and limb anomalies. Further investigation is required to completely define the pathogenesis of the hind-limb disruptions.

In conclusion, nitric oxide is a critical mediator of fetal growth and development, and deficits in nitric oxide production lead to fetal growth retardation and hemorrhagic necrosis of the hind limbs. However, maternal complications of L-NAME treatment do not mimic preeclampsia. Adequate, if not heightened, production of nitric oxide is essential for a successful outcome in pregnancy.

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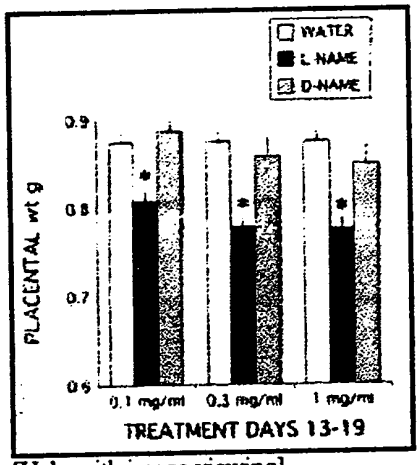

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Figure 3. Placental wet weights from untreated dams (control) or dams treated with L-NAME or D-NAME from gestational days 13 to 19 at 0.1, 0.3, or 1.0 mg/ml in drinking water. Columns represent mean +/-SEM. Asterisk, Significant difference in the L-NAME group compared with water or D-NAME controls ($p < 0.5$); n = 57 to 111 placentas per group per dose

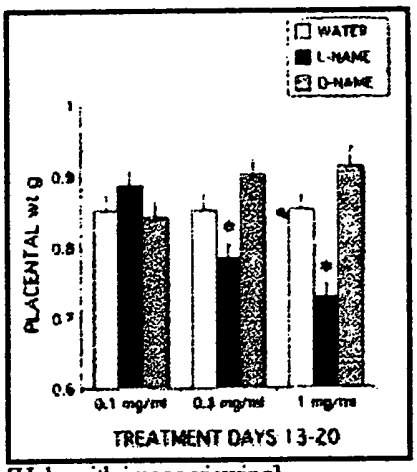

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Figure 4. Placental wet weights from untreated dams (control) or dams treated with L-NAME or D-NAME from gestational days 13 to 20 at 0.1, 0.3, or 1.0 mg/ml in drinking water. Columns represent mean +/-SEM. Asterisk, Significant difference in L-NAME group compared with water or D-NAME controls ($p < 0.05$); n = 54 to 116 placentas per group per dose

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